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Immunologic response of swine to solubilized outer envelope components of *Treponema hyodysenteriae*

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IMMUNOLOGIC RESPONSE OF SWINE TO SOLUBILIZED OUTER
ENVELOPE COMPONENTS OF TREPONEMA HYODYSENTERIAE

Iowa State University

PH.D. 1982

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Immunologic response of swine
to solubilized outer envelope
components of Treponema hyodysenteriae

by

Loren Harold Peterson

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
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For the Graduate College

Iowa State University
Ames, Iowa

1982

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ABSTRACT

The outer envelope of Treponema hyodysenteriae strain B204 was solubilized with CHAPS detergent, a zwitterionic derivative of cholic acid. At least two T. hyodysenteriae specific proteins were identified by agar gel double diffusion and SDS PAGE of immune precipitates using rabbit anti-T. hyodysenteriae serum absorbed over a Treponema innocens strain B256, a nonpathogen, affinity column.

A passive immunity study indicated swine dysentery recovered sows provided protection to their piglets via the milk up until weaning. An amplified ELISA detected IgA specific antibodies to partially purified outer envelope antigens. Levels of IgA antibodies to fractions containing T. hyodysenteriae specific proteins were associated with passive protection to swine dysentery.

Four groups of pigs were vaccinated intraperitoneally with preparations containing T. hyodysenteriae specific proteins. Although protection against swine dysentery was not complete, shedding of T. hyodysenteriae and onset of clinical signs in three of the groups were delayed.

INTRODUCTION

Swine dysentery is an enteric disease caused by an anaerobic bacterium, T. hyodysenteriae. This organism grows in the large intestine of the pig and causes a bloody diarrheal disease. Although in many instances the disease may be of limited severity with few deaths, the economic impact due to weight loss is of significant importance to the hog production industry.

It has been shown that pigs which naturally recover from the disease are resistant to subsequent challenge with live organisms. The actual mechanism of disease and development of resistance to swine dysentery are not known. While there is a wealth of published information regarding infections affecting the small intestine, less is known about infections of the large intestine. Research on the development of resistance by the host to this disease has relevance to our understanding colonic infections in other animals, including man. Recent evidence indicates there is a definite link between development of gut immunity and the onset of a secretory IgA response by the mammary gland.

Being essentially devoid of immunoglobulins at birth, the pig is a unique model for study of ontogeny of intestinal immunity. The pig has a relatively short gestation period with delivery of large litters. Thus, the pig is an ideal animal for passive immunity studies. Also, sow's milk is rich in IgA without the inherent collection and storage problems associated with intestinal secretions.

RESEARCH OBJECTIVES

- A) Characterize specific antigens from pathogenic strains of I. hyodysenteriae.
- B) Determine if resistance to swine dysentery is primarily cellular or humoral mediated.
- C) Develop reagents that could be used in the study of the humoral immune response to I. hyodysenteriae.
- D) Use these reagents to determine the classes or subclasses of immunoglobulins involved in protection.
- E) Determine the role purified antigens play in immune protection to the disease.

LITERATURE REVIEW OF SWINE DYSENTERY

Etiology

Swine dysentery is a widely occurring mucohemorrhagic diarrheal disease. Various names such as bloody scours, bloody dysentery, hemorrhagic dysentery, vibronic dysentery, black scours, or mucohemorrhagic diarrhea have been used to describe this disease. The etiologic agent has been shown to be an anaerobic spirochete, Treponema hyodysenteriae (Taylor and Alexander, 1971; Harris and Glock, 1972; Harris et al., 1972a).

Swine dysentery was first reported by Whiting et al. (1921). Subsequently, various reports implicated a comma-shaped microorganism, Vibrio coli as the etiologic agent (Doyle, 1944; James and Doyle, 1947; Carpenter and Larson, 1952). Using pure culture of V. coli, other workers were unable to reproduce the disease (Andress and Barnum, 1968; Andress et al., 1968; Harris et al., 1972a and 1972b). Also, V. coli was shown to be present in healthy pigs (Andress and Barnum, 1968; Andress et al., 1968; Terpstra et al., 1968; Harris and Glock, 1971 and 1972; Harris et al., 1972a), casting further doubt as to whether or not V. coli was the etiologic agent of swine dysentery. Terpstra et al. (1968) obtained serum from dysenteric pigs and conjugated the gamma globulin portion with fluorescein dye. The tagged immunoglobulin reacted with a spiral-shaped organism and not with V. coli.

Electron microscopy provided an insight into the causative agent when Taylor and Blakemore (1971) and Glock (1971) demonstrated a spirochete associated with the colonic tissue in affected swine. Taylor

and Alexander (1971) were able to culture this organism on horse blood agar and reproduce the disease in susceptible pigs. Harris et al (1972a) were also able to grow the organism in pure culture and reproduce the disease in specific pathogen-free pigs. These workers named the organism T. hyodysenteriae.

T. hyodysenteriae will readily produce dysenteric lesions in combination with other organisms (Meyer et al., 1974 and 1975; Harris et al., 1978; Whipp et al., 1979 and 1980). It was once thought that one or more anaerobes must be present along with T. hyodysenteriae in order for pathogenicity of the spirochete to be expressed. Recently, however, clinical and pathological signs of swine dysentery were observed in gnotobiotic pigs inoculated with T. hyodysenteriae (Whipp et al., 1982).

In 1972, Taylor described a nonpathogenic spirochete isolated from normal pigs that produced a weakly beta-hemolytic pattern on blood agar. Kinyon (1974) reported a similar isolate obtained from normal pigs and a puppy with enteritis. These weakly beta-hemolytic isolates did not produce signs of swine dysentery when reintroduced into specific pathogen-free swine (Kinyon et al., 1977). Kinyon and Harris (1979) named this isolate, which is morphologically indistinguishable from T. hyodysenteriae, Treponema innocens. Using DNA hybridization, Miao et al. (1978) found that T. hyodysenteriae was genetically unrelated to Treponema pallidum and that T. hyodysenteriae and T. innocens showed a 28% sequence homology.

Phages have been shown to be present in culture of T. hyodysenteriae (Ritchie et al., 1978). Although the existence of a plasmid in T. hyodysenteriae has not been shown, its presence appears quite likely, for

a plasmid DNA structure has been identified in T. pallidum (Nichols) (Norgard and Miller, 1981). Phages and a possible plasmid of T. hyodysenteriae could potentially transfer virulence factors to the organism.

Pathology

Swine dysentery is described as a catarrhal enteritis with hemorrhage, affecting only the large intestine. Lesions (Whiting et al., 1921; Taylor and Blakemore, 1971; Glock and Harris, 1972) consist grossly of ascites and a hyperemic, edematous colon. Blood is consistently found in the lumen. The mucosal surface often has a mucofibrinous exudate which frequently forms a pseudomembrane. At the light microscopic level, the mucosa and submucosa appear edematous with a mixed inflammatory response. The epithelium becomes completely eroded from the lamina propria in some areas and a mucofibrinous exudate covers the luminal surface. Colonic crypts are dilated with mucus and contain large numbers of spirochetes. Electron photomicrographs reveal these to be embedded in the apical surface of affected cells. Attachment and invasiveness of the organism, however, have not been established as being necessary for producing lesions. The observed diarrhea is due to colonic malabsorption occurring as a result of failure in mechanisms involving active transport of sodium and chloride from the intestinal lumen to the blood (Argenzio, 1980; Argenzio et al., 1980; Schmall, 1980).

Diagnosis

The differential diagnosis of swine dysentery from other diseases is essential (Harris and Glock, 1981). Clinical signs of diarrhea with

blood and mucus in the feces, depression, and dehydration are indicative but not pathognomonic of swine dysentery. At necropsy, lesions are limited to the large intestine. The accurate diagnosis of swine dysentery includes the isolation and identification of I. hyodysenteriae from feces or colonic mucosa.

Songer et al. (1976) developed a selective medium containing spectinomycin for the isolation of I. hyodysenteriae. He reported that the medium reduced contaminating flora by 99.9% and that the pathogenicity of I. hyodysenteriae was not affected. While I. hyodysenteriae forms a beta-hemolytic pattern, I. innocens produces a weakly beta-hemolytic pattern on selective blood agar plates. This medium has subsequently become a useful diagnostic tool (Kinyon et al., 1976). It is also possible to differentiate I. innocens from I. hyodysenteriae on the basis of lipid components (Matthews et al., 1980).

Treatment

Presently, treatment for swine dysentery includes the use of chemotherapeutics or antibiotics together with electrolytes. Arsenicals have been extensively used in prophylaxis and treatment but resistance by I. hyodysenteriae has developed (Harris and Glock, 1981). Other effective compounds used in the treatment or prophylaxis of swine dysentery include sulfonamides, nitrofurans, quinoxalines, mutilins, nitroimidazoles, and the following antibiotics: streptomycin, bacitracin, neomycin, tylosin, gentamycin, chlortetracycline, virginiamycin, and lincomycin (Harris and Glock, 1981).

Resistance

Susceptibility of swine to T. hyodysenteriae is thought to be dose related (Alexander and Taylor, 1969). Age appears to have little influence on resistance (Alexander and Taylor, 1969; Olson, 1974), although it is questionable whether nursing piglets are susceptible (Dr. D. L. Harris, Dept. of Vet. Micro., Iowa State Univ., Ames, Ia., personal comm., 1980). The incubation period for swine dysentery ranges from 2 days to 3 months, with 10 to 14 days being most common in naturally infected pigs (Harris and Glock, 1981).

Clinical signs of swine dysentery may appear in a cyclic manner in affected herds often following drug treatment. This observation has led many workers to incorrectly conclude that pigs are unable to develop resistance to swine dysentery (Harris and Glock 1981). Other workers (Olson, 1974; Joens, 1977; Joens et al., 1979a) have provided ample evidence that pigs naturally recovered from swine dysentery are immune to later challenge with T. hyodysenteriae. Joens et al. (1979a) demonstrated that untreated pigs recovered from swine dysentery seroconverted and were resistant to re-exposure up to 16 to 17 weeks after initial exposure.

Genho et al. (1971) reported limited immunity to swine dysentery when pigs were injected intramuscularly with immunoglobulins obtained from recovered swine. Schwartz and Glock (1976) demonstrated significant passive resistance in challenged pigs which received serum from hyper-immunized pigs. Hudson et al. (1974) were unable to detect resistance in swine orally inoculated with an attenuated strain of T. hyodysenteriae.

However, limited protection was achieved by immunization with a combination of oral and parenteral administration of inactivated T. hyodysenteriae (Hudson et al., 1976). In addition, Glock et al. (1976) provided limited protection against swine dysentery in pigs with the parenteral use of a formalin-killed suspension of T. hyodysenteriae.

It is possible for recovered pigs to be asymptomatic carriers of T. hyodysenteriae with the ability to transmit the organism for as long as 70 days (Songer and Harris, 1978). Asymptomatic carriers of T. hyodysenteriae have been implicated in the natural spread of the disease (Harris and Glock, 1981). Ronidazole and dimetridazole treatment will eliminate the carrier state (Songer and Harris, 1978).

Immunologic information concerning the recovered immune pig is limited, although Joens et al. (1978a) demonstrated micro-agglutination response to T. hyodysenteriae at 2 to 4 weeks after initial inoculation. Peak titers occurred at 4 to 7 weeks after inoculation.

Antigenic Characterization

Baum and Joens (1979b), using hot phenol and water, extracted a water-phase antigen from pathogenic T. hyodysenteriae isolates obtained from different geographic locations. The water-phase antigens were assayed for precipitin reactions in agarose gel with antisera produced in rabbits against whole cells. Four serotypes of pathogenic beta-hemolytic T. hyodysenteriae were observed. Baum (1978) and Baum and Joens (1979a) briefly described a T. hyodysenteriae specific antigen present in the phenol phase.

Indirect and direct fluorescent antibody tests have been developed

for the diagnosis of swine dysentery, but they lack sensitivity and specificity (Joens et al., 1978a, 1978b, 1979b). A microtiter agglutination test developed by Joens et al. (1978a), which uses whole cell antigen, has been shown to be more consistent and sensitive. Joens et al. (1982) also developed an enzyme-linked immunosorbent assay (ELISA), which uses a water soluble antigen from hot phenol and water extract of I. hyodysenteriae. Saheb et al. (1980) have purified and characterized hemolysin from I. hyodysenteriae.

LITERATURE REVIEW OF SWINE IMMUNOLOGY

Ontogeny of the Immune Response

The neonatal pig is a good experimental animal for immunologic study. The pig has a six-layered epitheliochorial placenta which is impermeable to immunoglobulin transfer to the fetus (Brambell, 1970). Being essentially devoid of immunoglobulins, the newborn pig is dependent upon maternal colostral immunoglobulins for protection.

Although it is generally accepted that transfer of antibody across the placenta does not occur in swine, Meyers and Segre (1963) claimed to have demonstrated antibodies in the serum of colostrum-deprived piglets from a sow that had been hyperimmunized with diphtheria and tetanus toxoids. The globulin fraction of the piglet serum had to be concentrated ten times before any detected hemagglutinin activity could be shown. Antigenic stimulation of the fetus in utero was ruled out because antibodies were demonstrated in the sera of pigs from a sow which had been passively immunized.

In a more extensive study, Chaniago et al. (1978) collected a total of 1,147 blood samples from fetuses at various stages of development and assayed them for the presence of immunoglobulins. Detectable levels of IgG or IgM were observed in only 24 samples, all of which were collected after the 60th day of gestation. This is the stage at which antigenic introduction in utero will stimulate fetal antibody production (Binns, 1973). Schultz et al. (1971) detected antibody production in the fetus following in utero antigenic stimulation around days 70-80 of gestation. This correlates well with a marked increase in number of fetal pig

lymphoid cells bearing surface immunoglobulins. Peripheral immunoglobulin-bearing lymphocytes have been detected in the fetal pig two weeks before term. Using specific antisera, both IgM and IgG surface globulins were detected (Binns et al., 1972). Immunologic memory occurs when antigens are injected after day 80 of gestation. Very little antibody is detected when antigens are introduced prior to day 60 (Binns and Symons, 1974).

Yabiki and Namioka (1976) detected three immunoglobulin classes in umbilical cord serum two days before the expected day of parturition. Using fluorescein tagged antisera they were able to detect immunoglobulin secreting cells in the placenta. They were unable to show transfer of antibody from a hyperimmunized sow to the placenta. This supports a theory proposed by Kim et al. (1966) that the immunoglobulins in umbilical cord serum do not come from the dam's serum, but may be produced by immunoblasts in the chorionic layer and secreted into the amniotic fluid. The immunoglobulins are then absorbed by the fetal gastrointestinal tract.

Kim (1979), using specific pathogen-free Minnesota miniature swine which were aseptically hysterectomy derived and colostrum deprived, found that there was an absence of immunoglobulins or antibody forming cells 3-5 days prior to birth. He questioned whether background or natural antibodies are produced spontaneously or whether ubiquitous environmental antigens stimulate their formation.

Sheep red blood cells will form rosettes with pig T lymphocytes (Binns, 1978). The appearance of rosette-forming lymphocytes occurs around 60-80 days of gestation, the same time that the fetal pig acquires

the ability to respond to antigenic stimulation. Immaturity of T cells prior to this time may explain the nonresponsiveness to thymus-dependent antigens used in other studies (Escajadillo and Binns, 1975).

The pig acquires the ability for graft rejection before parturition (Binns, 1967). Pig fetuses at days 60, 80, and 104 of gestation were injected intraperitoneally with an allogenic white cell suspension. Piglets exposed to transplantation homographs at day 60 of gestation showed immunologic tolerance while those injected at later times showed graft rejection.

Porcine Immunoglobulins

There are presently four known classes of immunoglobulins in the pig: IgG, IgA, IgM, and IgE. IgG is the predominant immunoglobulin in both serum and colostrum (Bourne, 1971). Two subclasses, an electrophoretically fast IgG₁ and a slow IgG₂ have clearly been identified (Metzger and Fougereau, 1967; Bourne 1971; Metzger and Houdayer, 1972). Two other subclasses IgG₃ and IgG₄, have been shown to exist in much lower concentrations than IgG₁ and IgG₂ (Kaltreider and Johnson, 1972; Dr. J. Rapacz, Dept. of Genetics and Dept. of Meat and An. Sci., Univ. of Wisconsin, Madison, Wi., personal comm., 1982).

Porcine IgA was first isolated from milk by Bourne (1969a). IgA present in porcine external secretions is composed of two molecular forms: 9.5S and 11.6S molecules. IgA present in serum exists mainly in two forms: a 9.3S dimer and a 6.4S monomer (Porter and Allen, 1972). Pig secretory IgA contains a covalently bonded secretory component and J chain (Porter, 1973a).

Porcine IgM is in a pentameric form and can be isolated from euglobulin precipitate of serum (Bourne, 1971). IgM has been shown to be an active serologic antibody in response to gram-negative bacteria (Franek et al., 1962).

There is evidence for the presence of IgE in swine (Dr. K. Ishizaka, School of Medicine, Subdepartment of Immunology, the Johns Hopkins University, Baltimore, Md., personal commun., 1982). Barratt (1972) passively transferred skin sensitivity to lungworms from infected pigs to uninfected pigs. He demonstrated an antibody fraction containing homocytotropic activity eluted with IgA on diethylaminoethyl (DEAE) cellulose. This homocytotropic activity was removed from pig serum by absorption with antisera directed against either porcine IgA or human IgE.

Physico-chemical properties of the porcine immunoglobulin classes are shown in Table 1. Concentrations of these immunoglobulins in body fluids are shown in Table 2.

The major immunoglobulin present in colostrum is 7S IgG₂ (Porter, 1973b). The rest of the immunoglobulins present in colostrum, in descending order of concentration, are IgG₁, IgA, and IgM. The total content of IgG falls off by 30-fold in the first week of lactation. The fall in colostrum IgA is not as dramatic as the fall in colostrum IgG and, consequently, IgA is the predominant immunoglobulin in milk (Bourne, 1969b; Porter et al., 1970). Colostrum immunoglobulins are derived mainly from the serum of the sow. In contrast, IgA of milk is mainly locally produced in the mammary gland (Bourne, 1976).

Table 1. Some properties of porcine immunoglobulins (Bourne, 1971).

	Sedimentation Coefficient	Nitrogen	Carbohydrate (μ moles per mg.)							Extinction Coefficient (E)
			Fuc	Man	Gal	Glc	GlcNAc	GalNAc	SA	
Serum IgG ₁	7S	14-6	9	65	17	13	56	6		13-7
Serum IgG ₂	7S	14-5	6	53	17	11	51	--	--	---
Serum IgM	19S	13-4	25	178	76	21	146	--	--	12-5
Serum IgA	7S; 8.95S	---	--	--	--	--	---	--	--	---
Milk IgA	9.5S; 11.6S	14-0	27	102	75	33	110	17	43	13-9

Table 2. Immunoglobulin levels in serum, colostrum, and milk (Bourne, 1971).

	Mean immunoglobulin concentration (Mg. per ml.) \pm S.E.				Total Serum and Whey Protein (mg. per ml.)
	IgG	IgG ₂	IgA	IgM	
Serum from pork pigs	18-31 \pm 0-67	12-41 \pm 0-48	1-44 \pm 0-12	3-15 \pm 0-19	70
Sow serum	24-33 \pm 0-94	14-08 \pm 0-49	2-37 \pm 0-20	2-92 \pm 0-18	70
Colostrum (0 hour)	61-80 \pm 2-44	40-29 \pm 1-66	9-66 \pm 0-59	3-19 \pm 0-21	196
Milk (24 hours)	11-83 \pm 4-82	8-04 \pm 3-21	3-76 \pm 0-99	1-79 \pm 0-31	41
Milk (2 days)	8-16 \pm 3-17	5-02 \pm 1-80	2-72 \pm 0-67	1-81 \pm 0-41	35
Milk (3-7 days)	1-91 \pm 0-64	1-31 \pm 0-32	3-41 \pm 1-01	1-17 \pm 0-23	33
Milk (8-35 days)	1-37 \pm 0-62	0-99 \pm 0-45	3-04 \pm 0-74	0-89 \pm 0-25	33

It is very important that the baby pig nurse in the first few hours of life. Bourne (1969b), studied the transition of mammary secretion from colostrum to milk during nursing. He found that there is a dramatic fall in the concentration of total colostral whey proteins within the first 24 hours following parturition. Fifty percent of this fall occurs within the first four to six hours. The concentration of albumin remains at a constant level throughout lactation, but that of pre-albumin, alpha-2 macroglobulin, and beta-globulin all initially show a steady rise, reaching a maximum level at 2 to 7 days postpartum. Gamma globulin is the predominant protein of colostrum. Its concentration, initially very high, rapidly declines within the first 24 hours postpartum. Colostral immunoglobulins IgG, IgA, and IgM are absorbed through the neonatal pig's intestine into the blood (Porter, 1969). Yaguchi et al. (1980) using an ammonium sulfate test found significant positive correlation between levels of serum gamma globulin and survivability of piglets. In the farrowing process, delivery of pigs occurs at an average of 15 minute intervals. Competition for mammary gland nipples, together with falling colostral proteins, may place first order of birth at a physiologic and immunologic advantage (Bourne, 1969b).

Porcine colostrum and milk contain trypsin inhibitor, which has been shown to be very effective in protecting immunoglobulins from proteolytic degradation (Laskowski et al., 1957). The level of trypsin inhibitor is highest in colostrum and rapidly falls off after seven days to a very low level (Bainter, 1973). This fall parallels the emergence of IgA as the predominant immunoglobulin in milk. IgA has been shown to be resistant

to degradation by proteolytic enzymes (Tomasi and Bienenstock, 1968). Jensen and Pedersen (1979) suggested that the levels of IgG and IgA in newborn nursing piglet serum may be influenced by immunoglobulin concentration in colostrum and by colostral trypsin inhibitor.

A selective process of IgG₁ absorption by the mammary gland is present in the bovine (Milstein and Feinstein, 1968), whereas in pig, IgG immunoglobulins are found to be nonselectively absorbed by the mammary gland (Curtis and Bourne, 1971).

Using peroxidase-labeled anti-swine immunoglobulin, Szeky et al. (1979) studied the absorption of colostrum by the pig gut at the electron microscopic level. Epithelial cells of the jejunum and the proximal two-thirds of the ileum were responsible for most of the absorption.

Gut Closure

Speer et al. (1959) determined the length of time after birth that the baby pig is capable of absorption of Escherichia coli antibodies from colostrum. A sensitized red blood cell hemagglutination test was used to detect antibodies in piglet serum. They found that gut closure followed a first order reaction rate with a half-life absorption efficiency of three hours. Insignificant amounts of antibody were absorbed by the baby pig beyond 24 hours of age.

Lecce and Morgan (1962) compared piglets that had nursed a sow, to piglets starved from birth, with respect to their ability to absorb a large molecule, polyvinylpyrrolidone (PVP). PVP has a molecular weight and osmotic properties similar to those of serum proteins, yet it is not subject to degradation by proteolytic enzymes. Nursing piglets lost

their ability to absorb PVP within 24 to 36 hours postpartum. Starved piglets were able to absorb PVP when 86 hours old, the longest time in this study in which a piglet could be kept alive without nutrition.

Payne and Marsh (1962) found that absorption of gamma globulin is directly proportional to the amount given, until a maximum level is reached when 60 ml of whole swine colostrum is administered. They found that gut closure occurred within 24 hours in piglets allowed to nurse immediately after birth or fed modified cow's milk. Using fluorescein-tagged gamma globulin from different sources (bovine, human, equine, and porcine) the absorption of heterologous gamma globulin was also observed.

Neonatal Immunologic Defense

Passively acquired immunoglobulins are essential for the neonatal pig's immunologic defense. Within 24 hours after birth, circulating immunoglobulin levels reach maximum concentrations (Porter and Hill, 1970; Curtis and Bourne, 1971).

Using ^{125}I labeled immunoglobulins, Curtis and Bourne (1973) compared the half-lives of colostral-IgG, IgA, and IgM in serum of newborn pigs. The half-life of colostral-IgA in newborn pig serum is 2 to 3 days, which is similar to the half-life of serum-IgA produced by the piglet. IgA production by the piglet during the first 7 to 12 days of life does not significantly add to colostral-derived serum-IgA. The half-life of colostral-IgG in piglet serum ranges from 6.6 to 22.5 days, also similar to serum-IgG half-life produced by the piglet. Likewise IgG synthesis by the young piglet during the first 2 to 3 weeks of life does not significantly add to serum-IgG levels. The half-life of colostral-IgM in newborn

pig serum is 2.5 to 3 days, while the half-life of piglet produced serum-IgM ranges from 3.5 to 6.5 days. Unlike production of IgA and IgG, production of IgM by the neonatal pig does in fact significantly add to the IgM circulatory levels in the first 10 to 12 days of life (Curtis and Bourne, 1973). The immunoglobulin level in piglet serum during the first days of life may be even higher than that in sow serum (Porter, 1969).

After birth the intestinal mucosa is bathed by immunoglobulins which are either maternal in origin or synthesized locally (Porter, 1973a, b). Porter et al. (1970) found that in the young piglet the rate of passage of passively acquired immunoglobulins through the intestinal lumen between nursing was slower than the normal passage rate of ingesta through the intestinal tract, thus providing additional protection. Rejnek et al. (1966) studied the effect of antibodies in the intestinal tract of germ-free baby pigs whose gut had been closed to macromolecules by pretreatment with modified cow's milk. Although no circulating anti-E. coli antibodies were detected in the serum of the experimental animals after the administration of serum or colostrum, antibodies in the colostrum protected the newborn piglets against E. coli challenge.

Brandenburg and Wilson (1974) showed that passively acquired antibodies from colostrum whey or IgG from vaccinated sows helped newborn piglets to overcome an artificially induced E. coli bacteremia. Lecce and Reep (1962) found that piglets with low initial serum immunoglobulin concentration were more prone to systemic E. coli infection. Chidlow and Porter (1978) showed colostrum-IgM anti-E. coli antibody produced significant in vitro complement-mediated bactericidal action and in vivo

bacterial elimination. Barta et al. (1970) and Rice and L'Ecuyer (1969) found colostrum-deprived piglets had low serum complement levels. Low levels of complement, together with a reduction in complement-fixing antibodies, places the baby pig at an immunologic disadvantage.

Colostrum-derived immunoglobulins are important elements in the respiratory immune response of neonatal pigs. Bradley et al. (1976), using horseradish peroxidase-anti IgA, were able to detect colostrum-derived IgA in the nasal mucosa of a 4-day-old piglet prior to the appearance of immunoglobulin-containing cells in the lamina propria.

An immune response by the piglet may be inhibited by passively acquired antibodies which delay the onset of active immunity (Muscoplat et al., 1977). These workers suggested that maternal antibodies regulate the immune response of the neonatal piglet through a process of antigen elimination and through a mechanism involving cytophilic antibody. They investigated the ability of maternal antibody to regulate the humoral immune response of neonatal piglets to sheep red blood cells (SRBC) and trinitrophenylated (TNP) sheep red blood cells (TNP-SRBC). Passively acquired maternal antibody inhibited an in vitro primary immune response to SRBC yet did not inhibit a TNP response. Their conclusion was that maternal antibody regulates the in vitro response only at the B cell level; T cell helper function could not have been inhibited or no anti-TNP response would have occurred following stimulation with TNP-SRBC.

Ontogeny of Gut Immunity

The intestinal tract of the newborn piglet receives significant antigenic challenge and is therefore extremely important in early development

of the immune response (Porter, 1975). Antigenic stimulation results in infiltration of lymphocytes into the lamina propria. This response is not observed in germfree pigs (Kenworthy, 1970).

Chapman et al. (1974) studied the embryonic development of intestinal lymphoid tissue and immunoglobulin-containing cells of 55 porcine fetuses. They were able to detect lymphoid cells in the Peyer's patches as early as 50 days of gestation. Their work supports a hypothesis that the lymphoid follicles present in Peyer's patches are mammalian bursa-equivalent sites.

Allen and Porter (1973) studied the distribution of IgM and IgA cells in intestinal mucosa and lymphoid tissue from nine unweaned 4-week-old pigs. They examined duodenum, jejunum, ileum, Peyer's patches, mesenteric lymph nodes, intercostal lymph nodes, spleen and thymus. These workers found as many IgM producing cells as IgA secreting cells in the lamina propria of the small intestine. IgA and IgM secreting cells were found predominantly around crypts with a few cells appearing in the cores of some villi. In all other tissues studied, however, there were proportionally more IgM cells present.

Inoue et al. (1978) measured the concentration of immunoglobulins in feces of piglets at 1 day to 10 weeks of age. The levels of IgG, IgA, and IgM and total immunoglobulin were significantly higher at 1 or 2 days of age than were those measured at 1 to 3 weeks of age. Total immunoglobulin content of fecal material was highest at 2 days of age, with 1 day of age being second. Immunoglobulin content of fecal material was least at 1 week to 3 weeks of age. IgG in piglet feces was 0.56 mg/g, 0.63 mg/g, and

0.17 mg/g at 1 day, 2 days, and 1 week of age, respectively. IgA in piglet feces at 1 day, 2 days, and 1 week of age was 7.29 mg/g, 23.58 mg/g, and 1.02 mg/g, respectively. IgM in piglet feces at 1 day and 2 days of age was 0.28 mg/g and 2.33 mg/g, respectively. IgM in feces at 1 week to 10 weeks of age varied between 0.09 mg/g and 0.02 mg/g. The amount of immunoglobulin found in piglet feces may be a reflection of the concentration of immunoglobulin present in sow's milk relative to the rates of absorption, degradation and interaction with intestinal flora, as well as production by the piglet.

Brown and Bourne (1976) studied the distribution of immunoglobulins and immunoglobulin containing cells in the intestinal tract, spleen, and mesenteric lymph nodes of pigs ranging in age from 12 weeks to 2 years. Using peroxidase labeled antisera against IgA, IgG, and IgM, they detected immunoglobulin containing cells in the lamina propria of the intestinal tract with all three antisera, with IgA containing cells being the most common. IgA and IgM cells were found in high numbers in the lamina propria of the stomach, duodenum, jejunum, and ileum, and to a lesser extent in the cecum, colon, and rectum. In the spleen, immunoglobulin secreting cells were present primarily in red pulp and peripheral white pulp, with IgM cells being somewhat more numerous than IgG or IgA cells. In the mesenteric lymph nodes, the number of IgA, IgM and IgG cells were approximately equal. They concluded that the pig's spleen and mesenteric lymph nodes appeared to have less of a role in the synthesis of immunoglobulins and that the gut-associated lymphoid tissue (GALT) may be the bursa-equivalent in the pig.

Bourne et al. (1971) reported that IgG, IgA, and IgM were present in the small intestinal washing from adult sows. IgA was the major immunoglobulin present with an average IgA:IgG ratio of 12:1. IgM was detected in most samples but at very low concentrations.

Mechanism of Intestinal Immunity

In 1919, Besredka demonstrated that oral immunization of rabbits provided protection from Shigella bacillus infection irrespective of the serum antibody level (Bienenstock, 1974). This was the first indication that the route of exposure of an antigen influences the type and location of antibody response. For example, Ogra et al. (1968) reported that oral immunization for polio with the Sabin vaccine resulted primarily in an intestinal IgA response. In contrast, parenteral immunization with the Salk vaccine did not induce intestinal IgA. Both vaccines produced a humoral response.

Similar results have been demonstrated in pigs. Bohl et al. (1972) reported that sows parenterally challenged with live attenuated transmissible gastroenteritis (TGE) virus developed high levels of antibodies in serum and colostrum, but the levels in milk fell markedly within a few days post-farrowing. In contrast, sows naturally or experimentally infected with virulent virus orally developed lower levels of antibodies in serum and colostrum, but maintained higher levels in milk. Vaccinated sows developed TGE titers of the IgG class, whereas naturally or experimentally infected sows developed TGE titers of the IgA class. Protective antibodies of the IgA class are needed in the intestinal tract for passive protection against the TGE virus. Muller-Schoop and Good (1975) showed

that antigen would stimulate T and B cells of Peyer's patches when given orally, but not when given parenterally.

Particulate and soluble antigens are able to penetrate the epithellium of the gastrointestinal tract either by pinocytosis or through intercellular spaces (Walker et al., 1974). Large molecules tend to pass via the lymphatics to mesenteric lymph nodes, whereas smaller molecules pass via the portal capillaries to the liver (Thomas and Jewell, 1979).

Primed lymphocytes have been shown to migrate into antigen-free intestinal tissue that has been grafted under the skin or kidney capsule (Moore and Hall, 1972; Parrott and Ferguson, 1974). It is now clear that antigen does not have to be present in the tissue for migration of primed cells to occur.

In species other than the pig, it has been shown that upon antigenic stimulation, T and B lymphocytes within the Peyer's patches migrate via the lymphatics to the mesenteric lymph nodes and enter into the blood circulation via the thoracic duct (Thomas and Jewell, 1979). Finally, these cells return to seed the lamina propria of the intestine and other excretory mucosal surfaces (Craig and Cebra, 1971). While in the circulation the primed lymphoid cells pass through the spleen, liver, and other organs of the lymphoid system. Prime lymphoid cells mature into either IgA-secreting plasma cells or T cells (Thomas and Jewell, 1979).

In the pig, the route of intestinal lymphocyte migration appears to be different. Bennell and Husband (1981a and b) found that lymphoblasts are diverted from porcine intestinal lymph, and may enter the blood circulation at the level of the mesenteric lymph node.

MATERIALS AND METHODS

Bacteria

Pure cultures of I. hyodysenteriae strains B204 (pathogenic, U.S.A.), P18A (pathogenic, England), and FW10 (avirulent nonpathogenic, England) and I. innocens strain B258 (U.S.A.) were grown by the procedure developed by Kunkel and Harris (1981). I. hyodysenteriae strains P18A and FW10 were kindly provided by Dr. R. J. Lysons, Dept. of Clin. Vet. Med., Univ. of Cambridge, Cambridge, England. The liquid medium consisted of pre-reduced aerobically prepared trypticase soy broth (BBL, Div. of Bioquest, Cokeysville, Md.), cysteine, sodium bicarbonate, yeast extract (Difco Labs., Detroit, Mi.), normal pig feces extract, and fetal bovine serum (Grand Island Biological Co., Grand Island, N.Y.). The organisms were propagated at 37⁰ C under a deoxygenated atmosphere consisting of 10% carbon dioxide and 90% nitrogen. The bacteria were harvested in log growth phase after 36 to 38 hours of incubation. The number of cells was determined using a Petroff and Hauser counter (C. A. Hausser Scientific, Blue Reil, Pa.).

Antigen Extraction

Cultures of I. hyodysenteriae and I. innocens were harvested by centrifugation for 30 minutes at 15,000 X G at 10⁰ C. Pellets were resuspended in approximately 40X volume of phosphate-buffered saline pH 7.2 and again centrifuged for 30 minutes at 15,000 X G at 10⁰ C. This step was repeated 3 times. A final pellet was suspended in approximately 10X volume of either 20 mM CHAPS (3[(3-cholamidopropyl) dimethyl-ammonio] 1-propanesulfonate, Calbiochem-Berhing Corp., La Jolla, Ca.) 0.1 M sodium

phosphate pH 7.2 or 1% Nonidet P40 (BDH. Gallard-Schlesinger Chem. Mfg. Corp., Carle Place, N.Y.) 0.15 M NaCl-0.01 M Tris-HCl buffer pH 8.0. The suspension was vortexed lightly and allowed to stand at room temperature for 2 hours. The suspension was then centrifuged for 30 minutes at 15,000 X G at 20⁰ C. The supernatant fluid was then collected and used immediately or stored at 4⁰ C.

Electron Microscopy

Bacterial samples were prepared for negative contrast electron microscopy using a spray technique described by England and Reed (1980). Briefly, 2 drops of a log phase growth culture of *T. hyodysenteriae* B204 or 2 drops of a CHAPS extract of *T. hyodysenteriae* B204 were mixed with 30 drops of water, 1 drop of 0.5% bovine serum albumin (BSA) and 4 drops of 2% potassium phosphotungstate. This suspension was placed in a nebulizer (#146, Ted Pella Co., Tustin, Ca.) and then sprayed onto collodion-backed carbon coated grids for examination in the electron microscope.

Isopycnic Centrifugation

A CHAPS extract of *T. hyodysenteriae* B204 was centrifuged through a preformed 10-40% (w/v) linear sucrose gradient. This gradient was prepared as follows: Sucrose (Fisher Scientific Co., Fair Lawn, N.J.) was dissolved in phosphate-buffered saline, pH 7.2, as 10%, 20%, 30% and 40% (w/v) stock solutions. Approximately 9.5 ml of each stock sucrose solution in order of increasing concentration were layered onto the bottom of a 39-ml Quick-Seal tube (Beckman Instruments, Inc., Palo Alto, Ca.), using an 18 gauge 4 inch cannula and a syringe. Tubes were gently placed in a horizontal

position and the sucrose was allowed to diffuse. After two hours at room temperature, the tubes were replaced in the upright position. Two-ml samples containing approximately 30 mg/ml of protein were layered onto the top of the tubes and the tubes were sealed as described by the manufacturer (Beckman Instruments, Inc.).

Samples were centrifuged, using a VTi50 vertical tube rotar in a L2-65B ultracentrifuge (Beckman Instruments, Inc.), for 18 hours at 170,000 X G at 20⁰ C. Upon the completion of the run, the contents of each tube were fractionated into 39 1-ml samples, by piercing the bottom of each tube and draining by gravity flow. A refractometer was used to determine the refractive index of sucrose in each fraction and buoyant density was calculated at 20⁰ C. The fractions were stored in sucrose at -20⁰ C until needed.

Protein Assay

Dye binding assay

Protein concentrations of detergent extracts were determined using a dye binding assay as described by Bradford (1976). Bovine serum albumin (BSA) (electrophoretic grade, Calbiochem-Behrins Corp., Ca.) was used as reference protein. Its initial concentration was determined by absorption at 280 nm, with an extinction coefficient of $E_{1\text{ cm}, 0.10\%}^{280\text{ nm}} = 0.6$. Coomassie Blue G-250 and phosphoric acid used in this assay were purchased from Eastman Kodak Co., Rochester, N.Y.

Ultra violet absorption

Determination of protein concentration of immunoglobulin preparations was performed using absorption at 280 nm, with an extinction coefficient

of $E_{280\text{ nm}}^{1\text{ cm}, 0.10\%} = 1.3.$

SDS PAGE

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS PAGE) was performed using the discontinuous, Tris glycine, buffer system described by Laemmli (1970). Gels were prepared in a SE 600 vertical slab unit, (Hoefer Scientific Instruments, San Francisco, Ca.). The running gels contained 10% (w/v) acrylamide plus 2.7% (w/v) bis-acrylamide and the stacking gels contained 4% (w/v) acrylamide plus 2.7% (w/v) bis-acrylamide. Spacers were used to set the gel thickness at 1.5 mm and a comb was situated to form 10 sample slots in a 2 cm stacking gel. Acrylamide (electrophoretic grade), N-N'-methylene-bis-acrylamide and N,N,N',N'-tetramethylethylenediamine (TEMED), were obtained from Eastman Kodak Co., ammonium persulfate and glycine from Fisher Scientific Co. SDS and 2-mercaptoethanol were purchased from Sigma Chem. Co., St. Louis, MO.

Treatment buffer containing 0.125 mM Tris-HCL pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol was added to an equal volume of each sample and the samples were then placed in a 100⁰ C water bath for 90 seconds. Samples were electrophoresed at a constant current of 60ma for approximately 2.5 hours at room temperature. The molecular weights of unknown proteins were determined using a plot of the relative mobility (Rf) vs. log molecular weight of known standards (Weber and Osborn, 1969). These molecular weight markers were obtained from Pharmacia Fine Chemicals, Piscataway, N.J. and used as the manufacturer described.

Gels were stained overnight at room temperature in a solution containing 0.125% Coomassie Blue R-250 (Sigma Chem. Co.), 50% (v/v) methanol and 10% (v/v) acetic acid. The gels subsequently were destained for two hours at room temperature in 50% (v/v) methanol-10% (v/v) acetic acid with a gentle rocking motion. The gels were then placed in 7% (v/v) acetic acid-5% (v/v) methanol for 5-8 hours and destained with gentle rocking motion at room temperature. The final destaining solution was changed several times before the gels were photographed.

Two Dimensional Gel Electrophoresis

Two dimensional gel electrophoresis was conducted as described by O'Farrell (1975) with slight modification. A CHAPS extract of *T. hyodysenteriae* B204 was isoelectrically focused in 130 X 3 mm ID tubes using a GT 3 tube gel electrophoresis unit (Hoefer Scientific Instruments). Twenty- μ l samples containing approximately 10 mg/ml of protein were applied to each tube. Urea (Fisher Scientific Co.) used in the focusing procedure was deionized with AG 501-X8D (Bio-Rad Labs., Richmond, Ca.) mixed bed ion exchange resin. Ampholines in the range of 5-8 and 3.5-10 were obtained from LKB Instruments, Inc., Rockville, Md.

Upon completion of isoelectric focusing, the gels were treated for 1 hour at room temperature with 0.0625 M Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and laid onto the top of a vertical SDS polyacrylamide slab gel. The composition of this SDS polyacrylamide running gel and stacking gel was described earlier. In this procedure, stacking gel extended from the running gel to the top of the glass plate and a sample comb was not used. The gels were solidified in place with 1% (w/v)

agarose (Agarose C, Pharmacia Fine Chemicals). A small trough was cut along the edge of the gel into the agarose and molecular weight markers were applied as described previously.

An untreated gel was cut into 5-mm slices and allowed to diffuse for 30 minutes into 1 ml of 0.05 M NaCl at room temperature and the pH of each slice was determined with a pH meter (Beckman Instruments, Inc.). Upon completion of electrophoresis in the second dimension, the gels were stained with Coomassie Blue R250 and destained with methanol-acetic acid as described earlier.

Ammonium Sulfate Precipitation of a CHAPS Extract

Saturated ammonium sulfate (SAS), filtered through a 22 um filter, was used to precipitate a CHAPS extract of T. hyodysenteriae B204 containing approximately 20 mg/ml protein. Increasing amounts of SAS starting with 14% (w/v) up to 50% (w/v) were slowly added to the CHAPS extract. This was allowed to stir for 20 minutes at room temperature, then centrifuged at 15,000 X G for 5 minutes at room temperature. The supernatant was collected each time for subsequent addition of SAS.

Antisera

New Zealand white rabbits, approximately six months old, were used for production of antisera. Antigens included IgA, IgM, IgG₁-rich, IgG₂-rich, NP-40 extract of T. hyodysenteriae B204, porcine colostrum and porcine serum. Initial immunization consisted of 2 ml of an antigenic preparation containing approximately 1-2 mg of protein emulsified in Freund's complete adjuvant (Difco Labs.), which was administered in the hind toe pads. Two weeks later rabbits were inoculated intradermally

in multiple sites on their backs with 2-4 mg of antigen preparation emulsified in Freund's incomplete adjuvant (Difco Labs.). Two to 3 weeks after the second inoculation the rabbits were bled and serum samples collected and stored at -20°C until needed. Rabbit anti-porcine Fab' 2 was kindly provided by Dr. J. E. Butler (Dept. of Microbiology, University of Iowa, Iowa City, Ia.).

Affinity Chromatography

Cyanogen bromide activated Sepharose 4BCL

A CHAPS extract of T. innocens B256, porcine IgG₁-rich, IgG₂-rich, and fetal porcine serum were covalently attached to Sepharose 4BCL (Pharmacia Fine Chemicals) as described by Cuatrecasas and Anfinsen (1971). Briefly, a water slurry of Sepharose 4BCL was activated with 1 g of cyanogen bromide (Sigma Chem. Co.) per 10 ml of Sepharose 4BCL (cake consistency). During the activation, a pH of 11 was maintained with 5 M NaOH and the temperature was maintained near 20°C with ice. After 10 minutes of activation the Sepharose 4BCL slurry was placed in a Buchner funnel and washed with a 3 X volume of cold 0.02 M sodium citrate buffer pH 6.5. Respective protein samples equilibrated against the same buffer were immediately added to the activated Sepharose 4BCL. This slurry was then placed on a roller rack at 4°C and mixed slowly overnight.

The next day the Sepharose 4BCL was washed and equilibrated in a 3 X volume of 0.15 M NaCl 0.5 M glycine-NaOH buffer pH 8.0 to inactivate unreacted sites. This slurry was again placed on a roller rack at 4°C

and mixed slowly overnight. The Sepharose 4BCL was then placed in a Buchner funnel and washed with a 3 X volume of 1 M NaCl-0.01 M Tris-3 mM EDTA buffer pH 8.3, 0.02% (GFB) and a 3 X volume of 0.15 M NaCl-0.5 M acetic acid buffer pH 3.5 (AAB). The Sepharose 4BCL was finally equilibrated in 0.01 M Tris-HCl-0.15 M NaCl buffer pH 7.9, 0.02% Na Azide (TBS) and 40-ml columns were prepared. Ten to 15-ml serum samples were cycled over the columns at room temperature and 2 hours later eluted with TBS. Between cycles absorbed protein was eluted with AAB, the columns were then regenerated with TBS and the serum samples were concentrated back to their original volumes.

Affi-Gel 10

A CHAPS extract of T. innocens B258 was coupled to Affi-Gel 10 (Bio-Rad Labs.) an N-hydroxysuccinimide ester of succinylated aminoalkyl Bio-Gel A support, according to the procedure suggested by the manufacturer. Essentially 12-ml of Affi-Gel 10 were washed with a 3 X volume of cold water followed by a 3 X volume of cold isopropanol. The gel was then immediately washed with a 3 X volume of cold 0.1 M Hepes (Sigma Chem. Co.) buffer pH 7.5. A protein sample equilibrated against 0.1 M Hepes buffer pH 7.5 was added and the mixture was slowly stirred at 4⁰ C overnight. Unreacted sites were inactivated with 0.5 M Tris-HCl buffer pH 8.0. The gel was washed with 3 X volume of 7 M urea-1 M NaCl and then with 3 X volume of 5 M MgCl₂. Finally, the gel was equilibrated with TBS and a 10 ml column was prepared. Five-ml serum samples were cycled over the column at room temperature and eluted with TBS. Between cycles absorbed protein was removed with 5 M MgCl₂, the column was regenerated with TBS, and serum

samples were concentrated back to their original volumes.

Protein Concentration

Protein samples were concentrated by ultrafiltration with a stirred cell, using a PM-30 membrane designed to retain molecules in excess of 30,000 daltons (Amicon Corp., Danvers, Ma.). All concentration was done at room temperature under nitrogen pressure of 50 psi.

Double Diffusion

Procedure I

A solution containing 2% (w/v) agarose, (Indubiose, Fisher Scientific Co.) and 3% (w/v) polyethylene glycol 6000 (PEG), (Sigma Chem. Co.) was prepared in 0.01 M sodium barbital buffer pH 7.2 and 2-ml of the solution were placed in a 3.5 cm diameter Petri dish (Falcon, Div. Becton Dickinson and Co., Oxnard, Ca.). PEG was used to enhance sensitivity, as described by Harrington et al. (1971) and Butler (1980). Seven wells 3 mm in diameter were cut in the agarose, 3 mm apart. Six of the wells were placed in a circular pattern, with the seventh well in the center. Precipitin bands were allowed to develop for 24 hours at room temperature and then photographed. Procedure I was used throughout this study unless otherwise stated.

Procedure II

Two percent agarose (w/v), (Indubiose, Fisher Scientific Co.) in TBS was layered on 8 X 9 cm glass plates. Wells 2 mm in diameter were cut in rows 4 mm apart. Precipitin bands were allowed to develop for 24 hours at room temperature and then photographed.

Preparation of Immunoprecipitates

Immunoprecipitates were formed using rabbit anti-T. hyodysenteriae B204, that had been repeatedly absorbed over T. innocens B256 cyanogen bromide activated Sepharose 4BCL and T. innocens B256 Affi-Gel 10 affinity columns, with CHAPS extract of T. hyodysenteriae B204 and CHAPS extract of T. innocens B256. Point of equivalence was estimated using agar gel double diffusion procedure II. Serum samples were initially incubated with the CHAPS extracts for 2 hours at 37⁰ C. Samples were then placed at 4⁰ C overnight. The next day the samples were centrifuged at 15,000 X G for 30 minutes at 10⁰ C. The supernatant was removed and the precipitin pellets were suspended in approximately 10 X volume of TBS. These samples were again incubated at 4⁰ C overnight. The next day the samples were recentrifuged, resuspended in TBS, and incubated at 4⁰ C as previously described. The next day precipitins were collected by centrifugation and suspended in TBS.

Crossed Immunoelectrophoresis

Crossed immunoelectrophoresis was conducted using the procedure described by Vestergaard (1973) and modified by Lum (1981). The gel used for the first dimension (2.5 X 8.5 cm) was prepared by pouring 2.5 ml of 1% (w/v) agarose (low Mr, Bio-Rad Labs.), 2% (w/v) PEG 6,000 in 0.081 M Tris-0.02 M Tricine-1% Triton X-100 buffer pH 8.6 (TTT, Bio-Rad Labs.) onto the hydrophilic side of a Multiphor plastic plate (LKB Instruments, Inc.). A 3 mm well for antigen was cut 2 cm from the cathodic edge and 6 mm from the second gel. Electrophoresis was conducted using a Multiphor flat bed electrophoresis system (LKB Instruments, Inc.) which cooled the

gel during electrophoresis. The antigen was electrophoresed at 10 volts/cm for 90 minutes at 10⁰ C in the first dimension.

For electrophoresis in the second dimension 1 ml of anti-I. hyodysenteriae was added to 7 ml of 1% (w/v) agarose (low Mr, Bio-Rad Labs.) in TTT-2% (w/v) PEG buffer. This was poured onto the rest of the plastic plate (7 X 8 cm) and electrophoresed at 1.5 volts/cm for 16 hours at 10⁰ C. The gel was overlayed with no. 577 filter paper (Schleicher and Schuell, Inc., Keene, N.H.) and paper towels and pressed 1 hour with 20 pounds of pressure. The pressed gel was then washed overnight in 0.1 M NaCl, briefly washed in H₂O, pressed again, dried and stained with 0.5% Coomassie Blue R250 in 45% (v/v) ethanol-10% (v/v) acetic acid. The gel was then destained in 45% (v/v) ethanol-10% (v/v) acetic acid, briefly washed in H₂O and photographed.

Immuno-electrophoresis

Immuno-electrophoresis was conducted in 2% (w/v) Noble agar (Difco Labs.) in 0.15 M sodium borate buffer pH 8.6 on glass slides. Troughs for antiserum 2 mm in width were cut the length of the plate. Wells for antigen 1.5 mm in diameter were cut 3 mm from the side of the troughs. Samples were electrophoresed with a current of 5 ma/cm. Electrophoresis was stopped after bromophenol blue dye (0.1% in 95% ethanol) used as migration indicator, had moved a distance of 3.5 cm. Rabbit anti-porcine immunoglobulin was used as the antiserum for the formation of precipitin patterns. Plates were photographed after developing for 25 hours at room temperature.

Gel Filtration

Immunoglobulin-rich fluid was passed through three 2.6 X 100 cm columns packed with Sepharose 6BCL (Pharmacia Fine Chemicals), as described by the manufacturer, and connected in series. Gel filtration buffer, 1 M NaCl-0.01 M Tris-3 mM EDTA buffer pH 8.3, 0.02% Na Azide (GFB), described by Butler et al. (1980c), was pumped through the columns in an upward direction with a flow rate of approximately 20 ml/hr. Blue dextran (Pharmacia Fine Chemicals) was used to determine the void volume, and immunoglobulin, BSA and cytochrome C (Sigma Chem. Co.) were used as molecular weight markers. Column elution was monitored by absorption at 280 nm and approximately 5-ml fractions were collected. Gel filtration and sample collection were performed at 4⁰ C.

Milk Collection

Sows were given 2-ml of oxytocin (20 U.S.P. units/ml, Holmes Serum Co., Inc., Springfield, Ill) intramuscularly to facilitate milk release during lactation. Approximately 10 minutes later sows were milked by hand. Milk was immediately centrifuged for 45 minutes at 15,000 X G at 10⁰ C. Milk whey supernatant was removed from the cream with a syringe and cannula and then frozen at -20⁰ C.

Isolation of IgA from Milk Whey

Milk whey was centrifuged in a L2-65B ultracentrifuge, type 30 rotor (Beckman Instruments, Inc.) for 1 hour at 170,000 X G at 10⁰ C. Whey supernatant was collected with a syringe and cannula. A pellet and a small amount of floating material was discarded. Forty ml of whey supernatant were precipitated with an equal volume of ammonium sulfate

added dropwise (50% (w/v) final concentration) with magnetic stirring action at room temperature. After 2 hours this material was centrifuged for 45 minutes at 15,000 X G at 10⁰ C. The precipitated pellet was suspended in 30 ml of 50% (w/v) ammonium sulfate. This material was recentrifuged for 45 minutes at 15,000 X G at 10⁰ C. The precipitate was dissolved in 20 ml of gel filtration buffer and centrifuged for 30 minutes at 15,000 X G at 10⁰ C. Ten-ml of the supernatant fluid was applied to Sepharose 6BCL for gel filtration.

Isolation of IgM from Serum

Serum from an approximately 4-month old pig was clarified by centrifugation for 30 minutes at 15,000 X G at 10⁰ C. Eighty ml of clarified serum were then dialyzed against 0.01 M sodium phosphate pH 7.2 for 2 days at 4⁰ C which included several buffer changes. The dialyzate was centrifuged for 1 hour at 15,000 X G at 10⁰ C. The precipitate was resuspended in 0.01 M sodium phosphate pH 7.2 and recentrifuged for 1 hour at 15,000 X G at 10⁰ C. This precipitate was suspended in 15 ml of GFB and centrifuged for 30 minutes at 15,000 X G at 10⁰ C. The supernatant fluid was collected and 11.5 ml were applied for Sepharose 6BCL gel filtration.

Removal of alpha-2 Macroglobulin

Alpha-2 macroglobulin was removed from IgA or IgM by an isoelectric point precipitation procedure described by Cambier and Butler (1974). Fractions constituting an IgA or IgM-rich peak were pooled, concentrated approximately 10 fold and dialyzed against 0.15 M NaCl-0.01 M sodium acetate buffer pH 5.4 overnight at 4⁰ C. This was then centrifuged for

30 minutes at 15,000 X G at 10⁰ C. Ten ml of 100 mM zinc sulfate (ZnSO₄)-0.15 M NaCl-0.01 M sodium acetate pH 5.4 were added dropwise, with magnetic stirring action at room temperature, to 10-ml of IgA or IgM-rich material. Two hours later this material was centrifuged for 30 minutes at 15,000 X G at 10⁰ C. IgA or IgM supernatant fluid was collected and chelated with 1% (w/v) EDTA.

Isolation of IgG from Serum

Serum from an approximately 4-month old pig was clarified by centrifugation for 30 minutes at 15,000 X G at 10⁰ C. Thirty ml of clarified serum were precipitated with an equal volume of ammonium sulfate added dropwise, 50% (w/v) final concentration, with magnetic stirring action at room temperature. After 2 hours, this material was centrifuged for 30 minutes at 15,000 X G at 20⁰ C. The precipitate was resuspended in 30 ml of 50% (w/v) ammonium sulfate. This material was recentrifuged for 30 minutes at 15,000 X G at 20⁰ C. The precipitate was equilibrated in 15 ml of GFB and centrifuged 15,000 X G for 10 minutes at 10⁰ C. Finally, 10 ml of supernatant fluid was applied to Sepharose 6BCL gel filtration. An IgG-rich peak was eluted, pooled and concentrated.

Anion Exchange Chromatography of a CHAPS Extract

A CHAPS extract of I. hyodysenteriae B204 containing approximately 35 mg/ml protein was equilibrated against 0.05 M NaCl-0.01 M Tris-HCl pH 8.3, 0.02% Na Azide. This was centrifuged for 5 minutes at 15,000 X G at room temperature. Five-ml of supernatant was applied to the top of a diethylaminoethyl (DEAE) Sephadex A50 (Pharmacia Fine Chemicals) column, 1.6 X 30 cm, equilibrated with the same starting buffer. A linear gradient

buffer, 0.5 M NaCl-0.01 M Tris-HCl pH 8.3, 0.02% Na Azide, was applied. Three-ml samples were collected at room temperature with gravity flow. Samples were assayed by conductivity and 280 nm absorption. Protein fractions were also assayed by the Coomassie Blue dye binding assay and individual peaks were pooled.

Anion Exchange Chromatography

Batch procedure

Five grams of DEAE Sephadex A50 (Pharmacia Fine Chemicals) were suspended in 0.05 M NaCl-0.01 M Tris-HCL-0.02% Na Azide buffer pH 8.3 (B-1). The pH of the DEAE Sephadex A50 was adjusted to 8.3. This was allowed to hydrate at room temperature for approximately 2 days. The DEAE Sephadex A50 was then equilibrated with fresh B-1 buffer. The ionic strength of the filtrate was equal to B-1 as determined by conductivity. A serum IgG-rich peak eluted from Sepharose 6BCL described earlier was concentrated approximately 5 X and equilibrated against B-1 overnight at 4⁰ C. This was centrifuged for 20 minutes at 15,000 X G at 20⁰ C. This IgG-rich supernatant fluid was collected, mixed with the B-1 equilibrated DEAE Sephadex A50, and allowed to stand at room temperature. After 2 hours, the DEAE Sephadex A50 was placed in a Buchner funnel and protein was eluted, in order of increasing ionic strength, with approximately 1 liter of each of the following buffers: B-1, 0.1 M NaCl-0.01 M Tris-HCl pH 8.3, 0.12 M NaCl-0.01 M Tris-HCl pH 8.3, 0.16 M NaCl-0.01 M Tris-HCL pH 8.3 and GFB. All buffers contained 0.02% Na Azide. Eluates were checked for ionic equilibration prior to addition of a successive buffer. Each eluate was concentrated approximately 200 X and stored at -20⁰ C.

Column procedure

DEAE Sephadex A50 was equilibrated in B-1 as described previously and a 1.5 X 90 cm column was prepared as suggested by the manufacturer. Five ml of IgG-rich supernatant (18 mg/ml protein) described previously was applied to the top of the column. B-1 buffer was used to elute the protein. Two-ml fractions were collected with downward gravity flow rate of 20 ml/hr at room temperature. Individual tubes were assayed by double diffusion prior to pooling.

Cellulose Acetate Electrophoresis

Serum proteins were electrophoresed on cellulose acetate membranes using a Beckman model R100 Microzone Electrophoresis System (Beckman Instruments, Inc.). Each sample, containing approximately 3 mg/ml of protein was applied 3 times. Electrophoresis was conducted with barbital buffer pH 8.6, 0.075 M, (Beckman Instruments, Inc.) at 250 volts for 30 minutes. Membranes were stained with Ponceau red dye (Beckman Instruments, Inc.) for 10 minutes and excess dye was removed by washing with 5% (v/v) acetic acid.

Amplified ELISA

An amplified enzyme-linked immunoassay (a-ELISA) as described by Butler et al. (1978), was performed with some modification. For convenience and conservation of reagents, the assay was conducted using Gilford Cuvette-Pak, a 10 well cuvette strip system, (Gilford Instrument, Oberlin, OH.). All buffers used in this assay were prepared as described by Engvall and Perlmann (1972) with antibody and wash buffers containing the nonionic detergent polyoxyethylene (20) sorbitan monolaurate, Tween 20 (J. T. Backer

Chem. Co., Phillipsburg, N.J.) to reduce hydrophobic interactions.

Wells were coated for 3 hours at 37⁰ C with 300 ul of antigen containing approximately 500 ng/ml of protein. Between steps, wells were washed 3 times to remove unbound protein. Plates were then covered with plastic to prevent evaporation and dust contamination. Wells were filled with 300 ul of a dilution of antibody and allowed to react at room temperature. A 1:1000 dilution of primary antibody was allowed to react overnight, in all other steps the reaction lasted for 2 hours. Goat anti-rabbit IgG bridging antiserum was kindly supplied by Dr. J. E. Butler (Dept. of Microbiology, Univ. of Iowa, Iowa City, Ia).

A rabbit immune complex of alkaline phosphatase, (calf intestinal mucosa type IV, Sigma Chem. Co.) was prepared and its optimal dilution together with that of secondary and bridging antibody were determined as described by Butler et al. (1980a and 1980b) and Butler (1981). Finally, 100 ul of substrate p-nitrophenyl phosphate (Sigma Chem. Co.) were added to each well. The reaction within a given plate or set of plates was stopped uniformly with the addition of 100 ul of 1 N sodium hydroxide (NaOH). Reaction time was determined empirically by visual observation of color development. Plates were read at 0. D. 405 nm absorption, using a spectrophotometer (Gilford PR-50 EI Analyzer Gilford Instruments, Oberling, OH.). Samples were run in triplicate and a mean value was obtained. In the event of an outlier (readings $S > 0.10$) were eliminated and the remaining two readings were averaged.

Sow Passive Immunity Study

Ten first litter gilts, all in the same stage of gestation and with no known history of swine dysentery were obtained from Pig Improvement Co., Spring Green, WI. The gilts were randomly assigned numbers 1 through 10. Six weeks prior to farrowing feed was withheld from all gilts. During the following 2 days, gilts 1 through 6 were given a daily intragastric inoculation of I. hyodysenteriae B204, consisting of 200 ml of 2.0×10^9 organisms per ml, via a stomach tube.

Gilts 7, 8, 9 and 10 housed as pairs in separate facilities, were given daily intragastric inoculations with 200 ml of growth medium via a stomach tube on consecutive days. After the second inoculation, all gilts were fed. Clinical signs of swine dysentery were observed and recorded daily. Rectal swabs were taken from all gilts 3 times a week and inoculated onto trypticase soy agar (BBL) containing 5% (v/v) citrated bovine blood agar 400 ug/ml spectinomycin (Upjohn Co., Kalamazoo, MI.) as described by Songer et al. (1976). The agar plates were incubated anaerobically at 42° C and observed at 2-day intervals for presence of beta-hemolysis typical of I. hyodysenteriae.

Gilts 1-6 were allowed to recover naturally from swine dysentery. Twenty-five days after the second inoculation the gilts' cages and rooms were cleaned and disinfected. All gilts were given 0.025% dimetridazol (Emtryl, Salsbury Labs., Charles City, Ia.) in the water ad libitum for 6 days. Approximately 1 week later, all gilts farrowed naturally. Colostrum and milk samples were periodically collected. At approximately 2 weeks of age, piglets from gilts 1 through 8 were challenged with I.

hyodysenteriae B204 while nursing. The intragastric inoculum consisted of 100 ml (1.0×10^9 organisms per ml) given via a stomach tube. Clinical signs of swine dysentery were recorded daily and rectal swabs were taken 3 times a week.

Vaccination Study

In this experiment, 20 pigs were randomly divided into 5 groups with each group containing 4 pigs. The pigs were purchased from Indian Creek Co., Ames, IA., a herd with no known history of swine dysentery. The pigs were castrated males which weighed an average of 37.6 pounds and ranged in weight from 31 to 47 pounds. Feed and water were supplied ad libitum.

On day one, all pigs in every group were inoculated intraperitoneally with a given antigen or saline emulsified with Freund's complete adjuvant. In group one, the control group, each pig received 2 ml of phosphate-buffered saline pH 7.2. In group two, each pig received 2 ml of CHAPS extract of T. hyodysenteriae, containing approximately 3 to 4 mg/ml of protein. In group three, each pig received 2 ml of fraction 3 and 4 after isopycnic centrifugation of a CHAPS extraction from T. hyodysenteriae, containing approximately 0.5 mg/ml of protein. In group four, each pig received 2 ml of fraction 12 and 13 after isopycnic centrifugation of a CHAPS extract of T. hyodysenteriae, containing approximately 0.5 mg/ml of protein. Each pig in group five received 2 ml of a rabbit anti-T. hyodysenteriae immunoprecipitin. This complex was prepared using a CHAPS extract from T. hyodysenteriae B204 and affinity purified rabbit antiserum against T. hyodysenteriae. The complex contained approximately 2 mg/ml of protein.

Three weeks later each pig within a group was inoculated intraperitoneally with identical antigen preparations as before except this time antigens were emulsified in Freund's incomplete adjuvant. Nine days prior to challenge feed was withheld from the pigs. During the following two days, all pigs were given a daily intragastric challenge of I. hyodysenteriae B204 consisting of 50-ml of 1.0×10^9 organisms per ml, via stomach tube. After the second challenge, all pigs were fed. Clinical signs of swine dysentery were recorded daily. Rectal swabs were taken throughout the course of the experiment from all pigs 3 times a week and cultured for I. hyodysenteriae as described earlier. Blood samples were also periodically obtained. Serum samples were collected and stored at -20°C until needed.

RESULTS

Antigenic Characterization

Figure 1 is an electron micrograph of I. hyodysenteriae B204 prior to extraction with CHAPS detergent. The organism has a thick outer envelope with axial filaments (flagella) inserted at each of its poles. Figure 2 is an electron micrograph of a CHAPS extract of I. hyodysenteriae B204. This extraction process apparently removed the outer envelope and flagella while leaving the protoplasmic cylinder intact. Visible are large portions of the outer envelope that have formed spherical vesicles. Strands of free flagella and aggregated particles are also present.

A typical graph of a CHAPS extract of I. hyodysenteriae B204 following isopycnic centrifugation is shown in Figure 3. Most of the extracted material, as detected by a dye binding assay, reached equilibrium between 1.180 and 1.060 g/ml buoyant density.

Figure 4 is a photograph of the electrophoretic pattern after SDS PAGE of fractions obtained by isopycnic centrifugation of a CHAPS extract of I. hyodysenteriae B204. The detergent-extraction process removed a heterogeneous population of proteins with a wide range of molecular weights. Isopycnic centrifugation resolved this extract into subpopulations of proteins of restricted molecular weight.

Figure 5 is a photograph of the electrophoretic pattern obtained after SDS PAGE of precipitates formed following the addition of an increasing concentration of ammonium sulfate to a CHAPS extract of I. hyodysenteriae B204. As the ammonium sulfate concentration increased,

Figure 1. An electron micrograph of T. hyodysenteriae B204 (10,000 X magnification).

Figure 2. An electron micrograph of a CHAPS extract of T. hyodysenteriae B204 (30,000 X magnification).

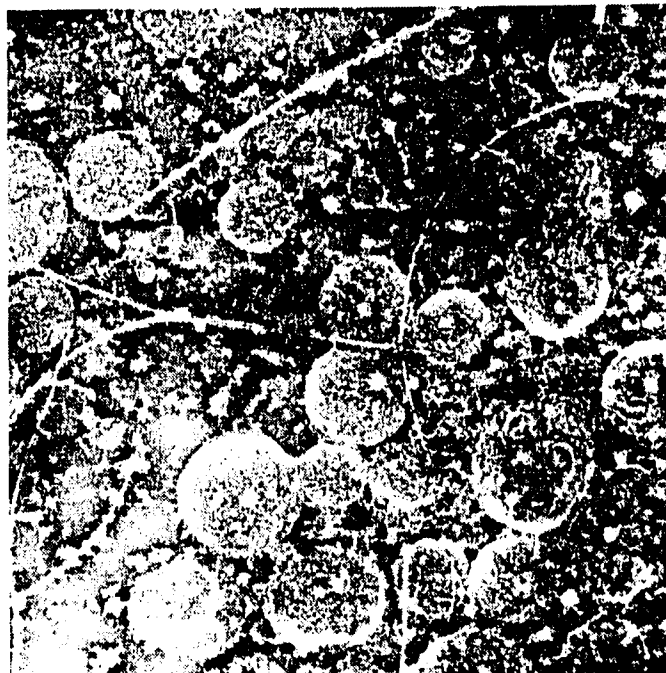
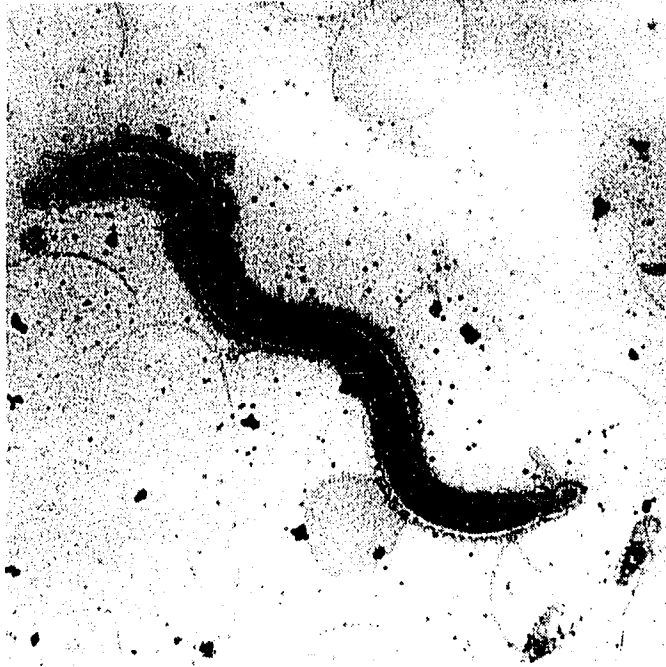


Figure 3. A graph of a fractionation pattern obtained from an isopycnic centrifugation of a CHAPS extract of T. hyodysenteriae B204 through sucrose. Buoyant density values are on the left (solid circles) and protein values are on the right (open circles). Tube fraction numbers starting from the bottom of the tube are presented in the lower portion of the graph.

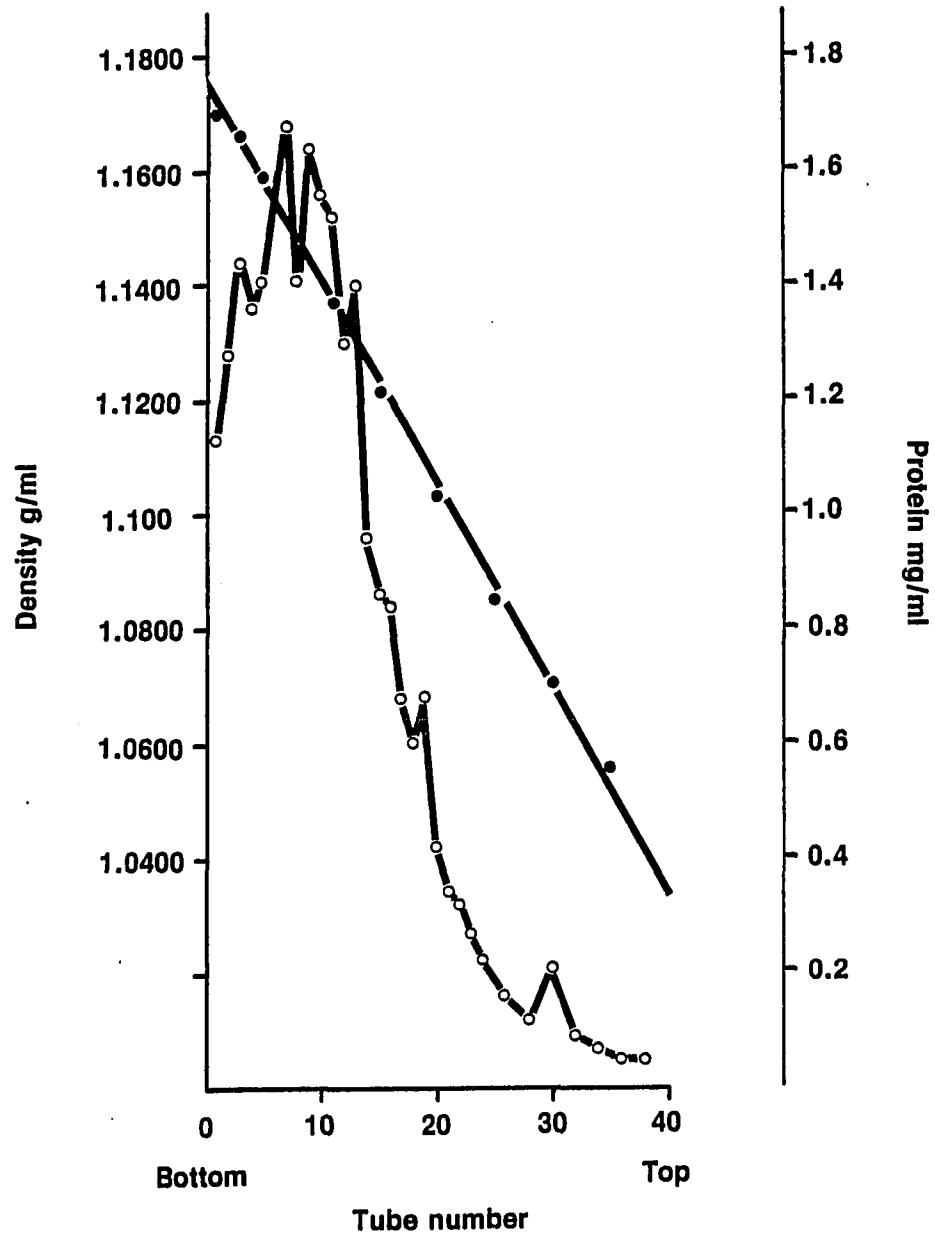


Figure 4. A 10% T 2.7% C SDS slab gel containing fractions from isopycnic centrifugation of a CHAPS extract of T. hyodysenteriae B204. Values of molecular weight markers, in thousands of daltons, are shown on the left. A CHAPS extract of T. hyodysenteriae B204 prior to centrifugation is shown in well Th followed by isopycnic centrifugation fractions 1 through 22.

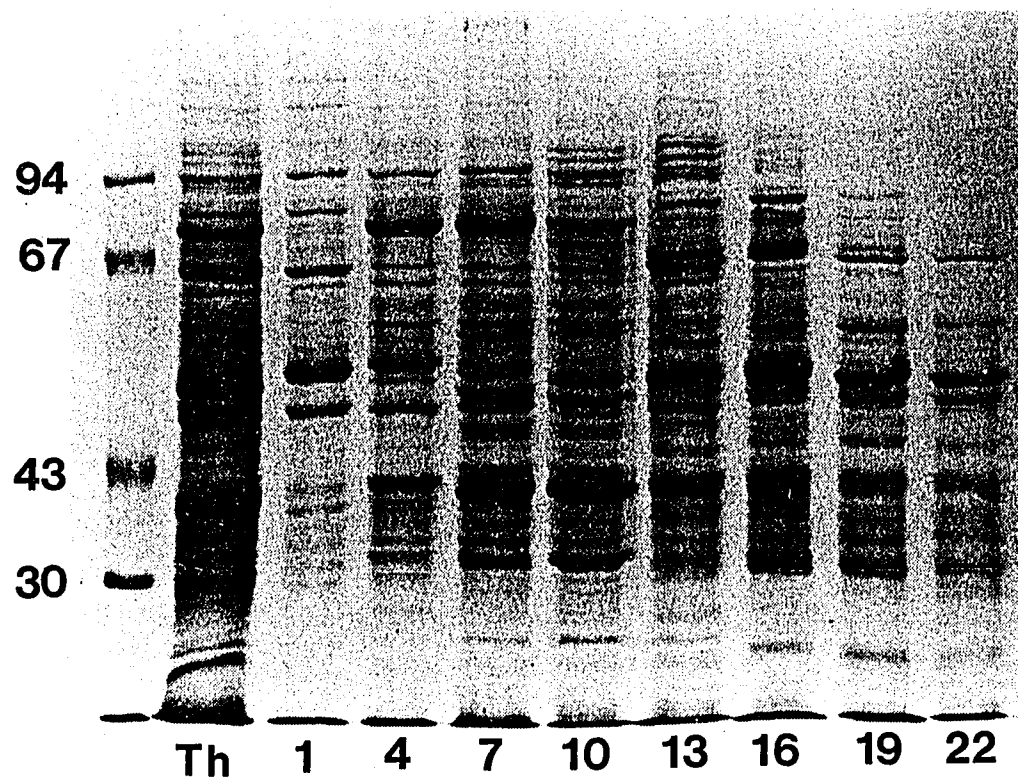
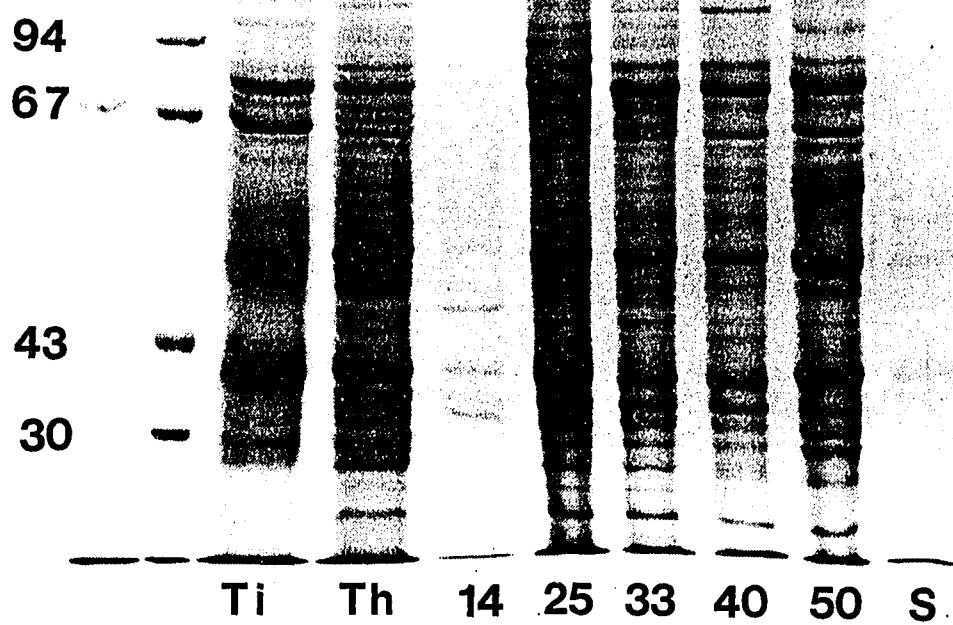


Figure 5. A 10% T 2.7% C SDS slab gel containing precipitates formed following the addition of an increasing concentration of ammonium sulfate to a CHAPS extract of T. hyodysenteriae B204. Values of molecular weight markers, in thousands of daltons, are shown on the left. Bovine serum albumin (BSA) is shown in the first well followed by the molecular weight markers, CHAPS extracts of T. innocens B256 (Ti), and T. hyodysenteriae B204 (Th), and ammonium sulfate precipitate 14% through 50% SAS. The final well contained 50% SAS supernatant.



more proteins were precipitated with almost all of the proteins being precipitated at 50% (w/v) ammonium sulfate concentration.

Figures 6a and 6b are photographs of immunoprecipitates formed with rabbit anti-T. hyodysenteriae serum before and after absorption over T. innocens B256 affinity columns with T. hyodysenteriae B204 and T. innocens B256. As shown in Figure 6a, prior to absorption this antiserum formed several precipitin lines with both T. hyodysenteriae B204 and T. innocens B256. Present are fewer lines of nonidentity. Prior to absorption, this antiserum agglutinated both T. hyodysenteriae B204 and T. innocens B256. After affinity absorption, this antisera no longer agglutinated T. innocens B256 and as shown in Figure 6b, many shared precipitin bands were removed. At least two T. hyodysenteriae specific precipitin lines remained.

Figure 7 is a photograph of the pattern obtained after crossed immunoelectrophoresis of a CHAPS extract of T. hyodysenteriae B204 with affinity-absorbed rabbit anti-T. hyodysenteriae. Several different precipitin arcs were separated by electrophoretic migration which could not be as clearly resolved by agar gel double diffusion.

Figure 8 is a photograph of the electrophoretic patterns obtained after SDS PAGE of immunoprecipitates formed with affinity absorbed rabbit anti-T. hyodysenteriae B204 serum and a CHAPS extract of T. hyodysenteriae B204 or T. innocens B256. Using this antiserum in agar gel double diffusion specific T. hyodysenteriae proteins were detected in fraction 3 and 13 (buoyant density 1.165 g/ml and 1.130 g/ml) obtained following isopycnic centrifugation.

Figure 6a. Agar gel double diffusion of rabbit anti-T. hyodysenteriae before absorption over a T. innocens B256 affinity column (well A), with a CHAPS extract of T. hyodysenteriae B204 (well B) and a CHAPS extract of T. innocens B256 (well C).

Figure 6b. Agar gel double diffusion of rabbit anti-T. hyodysenteriae after absorption over a T. innocens B256 affinity column (well A), with a CHAPS extract of T. hyodysenteriae B204 (well B) and a CHAPS extract of T. innocens B256 (well C).

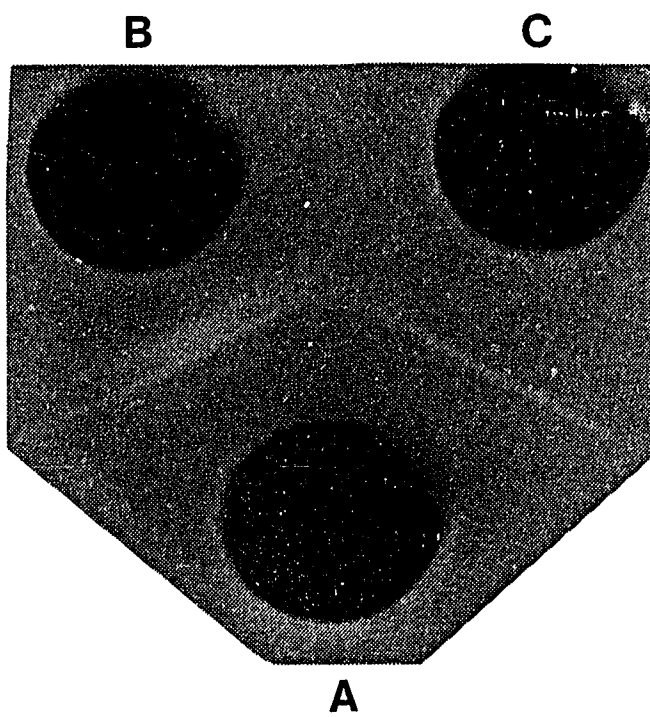
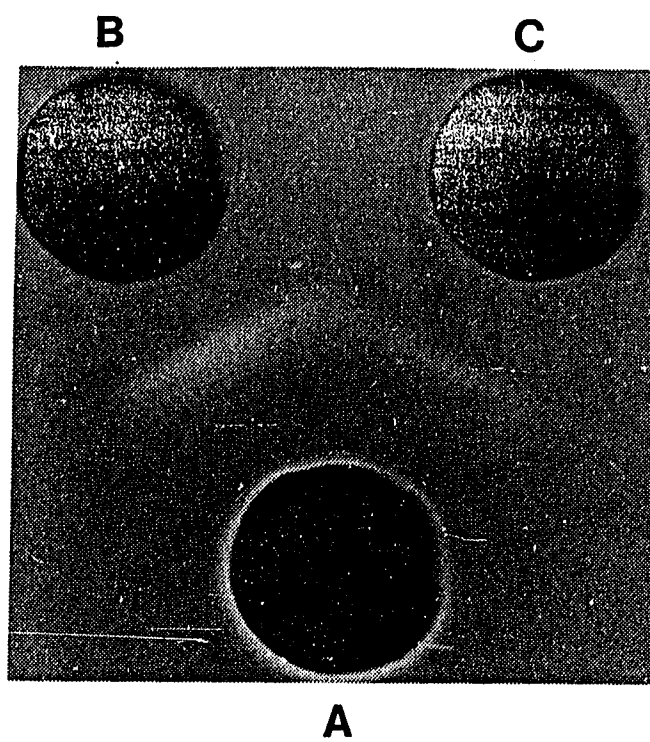


Figure 7. Crossed immunoelectrophoresis of a CHAPS extract of I. hyodysenteriae B204 with partially affinity-absorbed rabbit anti-I. hyodysenteriae B204. The anode is at the right.

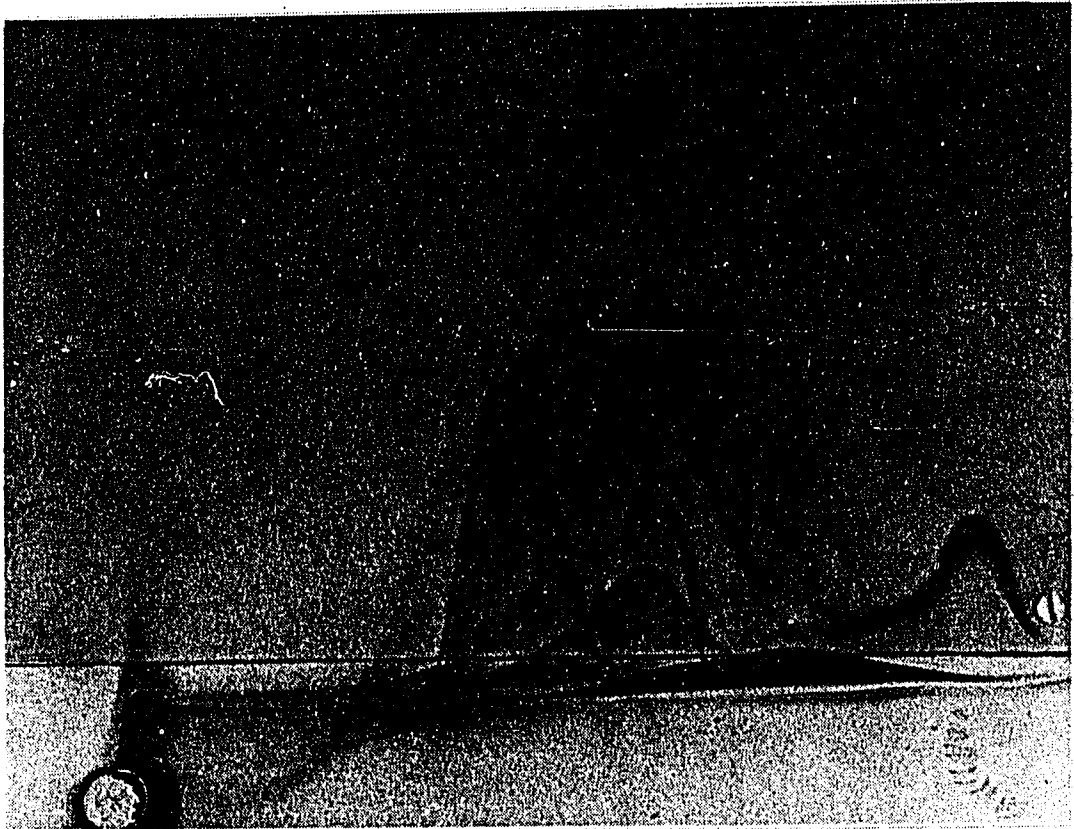
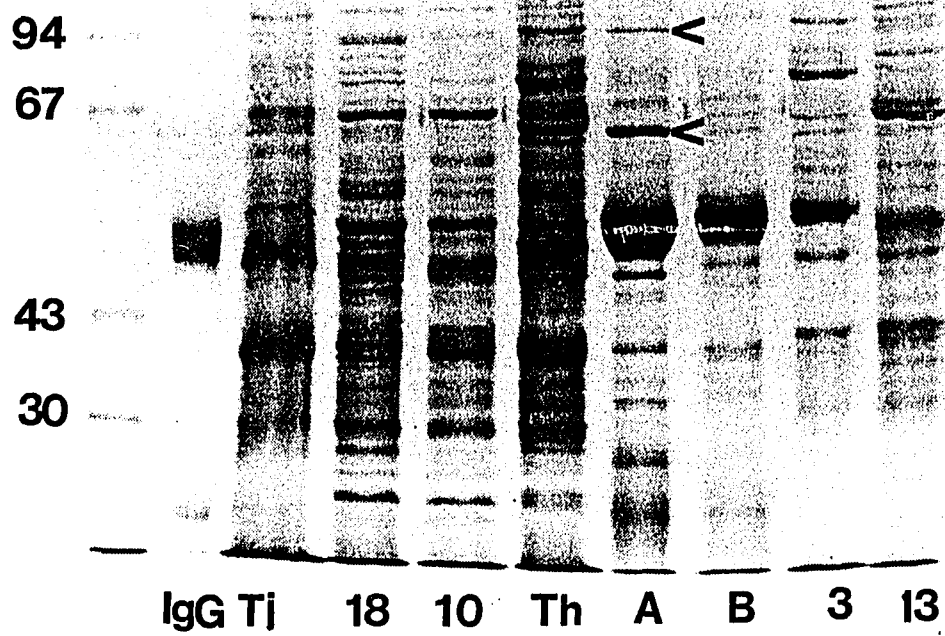


Figure 8. A 10% T 2.7% C SDS slab gel containing immuno-precipitates of CHAPS extract of T. hyodysenteriae B204 and T. innocens B256. Values of molecular weight markers, in thousands of daltons, are shown on the left. The wells contain from left to right: molecular weight markers, rabbit IgG, CHAPS extracts of T. innocens B256 (Ti), T. hyodysenteriae P18A (18), T. hyodysenteriae Fw10 (10), and T. hyodysenteriae B204, T. hyodysenteriae B204 immunoprecipitate (A), T. innocens B256 immunoprecipitate (B), isopycnic centrifugation fractions 3 (buoyant density 1.165 g/ml) and 13 (buoyant density 1.130 g/ml). T. hyodysenteriae specific proteins of molecular weight of 90,000 and 63,000 daltons are indicated.



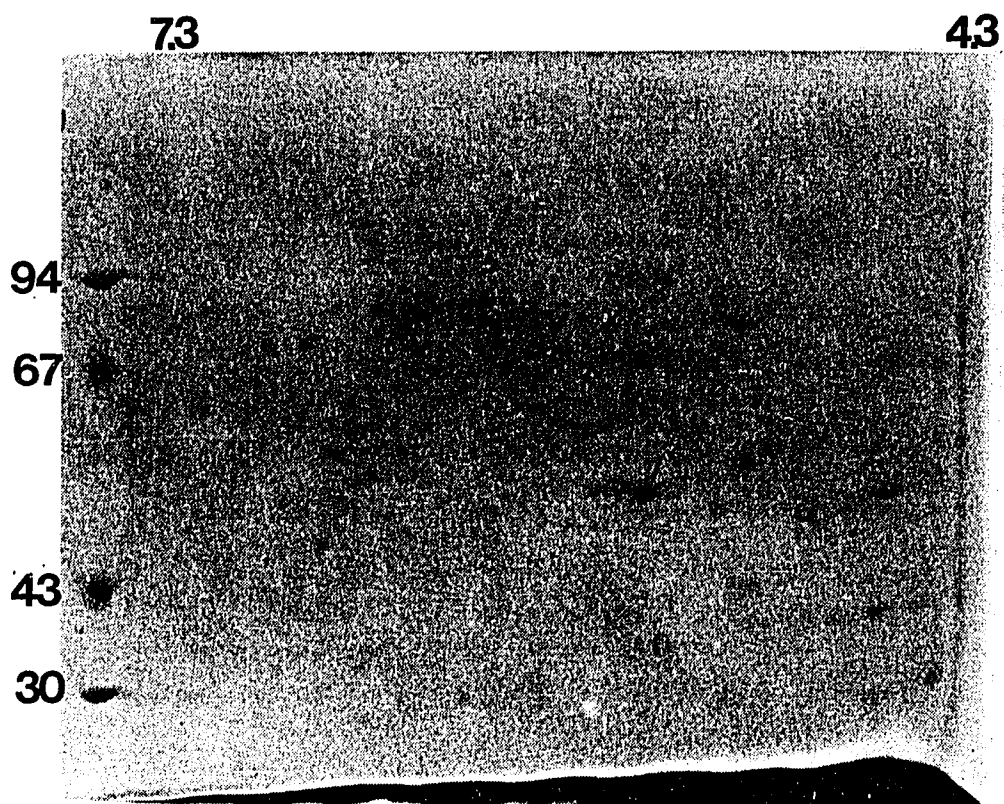
This antiserum precipitated components of a CHAPS extract of T. hyodysenteriae B204 that were not precipitated from a CHAPS extract of T. innocens B256. T. hyodysenteriae specific bands of protein were identified in the T. hyodysenteriae immunoprecipitate. They included one protein with a molecular weight of 90,000 daltons and another with a molecular weight of 63,000 daltons. The 90,000 dalton protein was present in isopycnic centrifugation fraction 3 (buoyant density 1.165 g/ml) and fractions T. hyodysenteriae P18, and B204. The T. hyodysenteriae specific protein with a molecular weight of 63,000 daltons was identified in isopycnic centrifugation fraction 13 (buoyant density 1.130 g/ml) and also in the CHAPS extract T. hyodysenteriae B204. A possible T. hyodysenteriae specific band could not be resolved because it was partially hidden by the heavy chain of the rabbit immunoglobulin.

Two dimensional gel electrophoresis was performed on a CHAPS extract of T. hyodysenteriae B204 as shown in Figure 9. Using this procedure, it was possible to simultaneously determine individual molecular weight and isoelectric point (pI) of some of the peptides extracted from T. hyodysenteriae. Although discrete spots of protein were detected throughout the gel, most had a molecular weight in the range 1-40,000 daltons and a pI less than 6.5.

Immunoglobulin Reagent Preparation

Gel filtration was used as an early purification step in the isolation of classes and subclasses of porcine immunoglobulins. The elution profiles of various fluids used in the isolation of IgG, IgM and IgA are shown in Figure 10a, b, c. The elution profile of IgG following an

Figure 9. A two dimensional 10% T 2.7% C SDS slab gel electrophoresis of a CHAPS extract of T. hyodysenteriae B204. Values of molecular weight markers are shown on the left, in thousands of daltons, and the pH values are shown on the top.



ammonium sulfate precipitation of serum is shown in Figure 10a. The shaded peak contained monomeric IgG. Pentameric IgM was isolated by gel filtration of an euglobulin precipitate of porcine serum. The elution profile is shown in Figure 10b. Pentameric IgM eluted in the first shaded peak. The elution profile of globulins following an ammonium sulfate precipitation of milk whey is shown in Figure 10c. This served as a source of dimeric IgA which eluted in the second shaded peak. The three classes of immunoglobulins were identified following elution by agar gel double diffusion. Homogeneous fractions were pooled, concentrated and further purified.

Immunoelectrophoresis was also used to identify the immunoglobulin classes. Figure 11 is a photograph of an immunoelectrophoretic precipitin pattern of normal porcine serum and of IgM, IgA, and IgG obtained by gel filtration. Rabbit anti-colostrum serum precipitated several normal proteins. The position and pattern of the precipitins formed with the isolated proteins were characteristic of IgM, IgA, and IgG. The limited migration of IgM as compared with the greater migration of IgA and still further migration of IgG is quite evident.

Repeated affinity absorption of serum from rabbits immunized with porcine IgA, IgM, and IgG₂ over alternate class and subclass affinity columns removed shared immunoglobulin precipitin activity. A fetal pig column removed extraneous serum protein activity from anti-IgA and IgG₂ serum.

A serum IgG-rich fraction was further resolved by anionic exchange chromatography into immunoglobulins of restricted electrophoretic

- Figure 10a. Gel filtration of an SAS cut of serum through Sepharose 6BCL. Monomeric IgG eluted in the third, shaded peak.
- Figure 10b. Gel filtration of an euglobulin precipitate of serum through Sepharose 6BCL. Pentameric IgM eluted in the first, shaded peak.
- Figure 10c. Gel filtration of a SAS cut of milk whey through Sepharose 6BCL. Dimeric IgA eluted in the second, shaded peak.

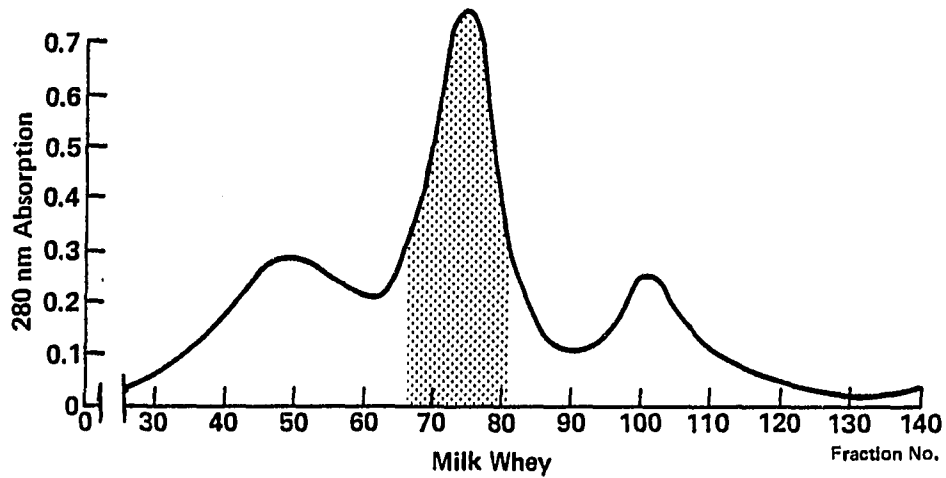
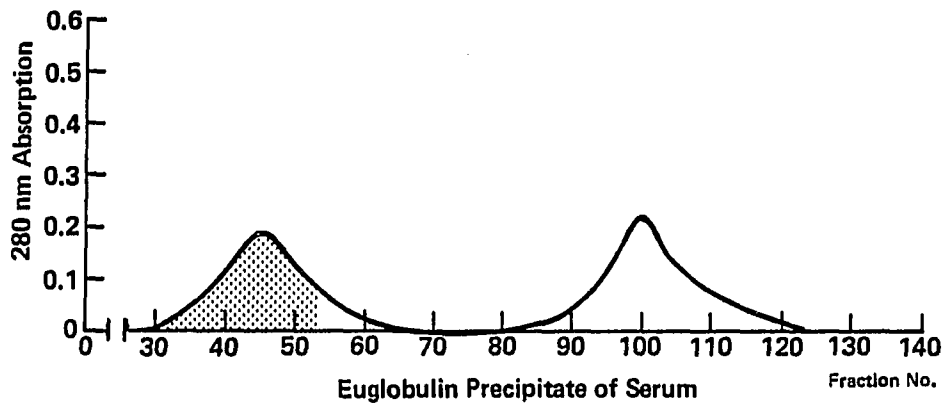
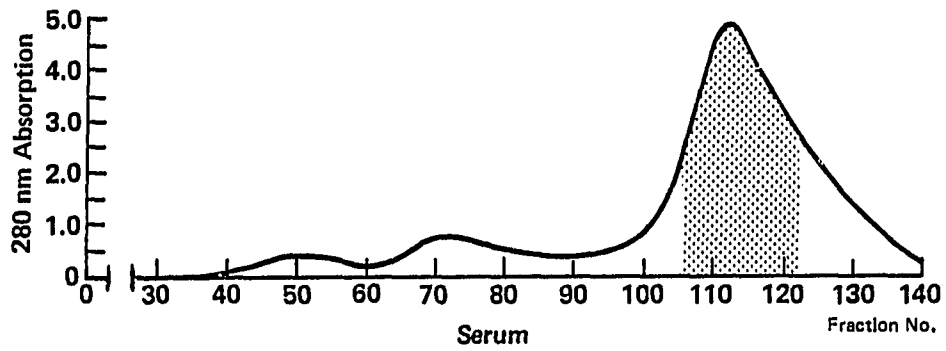
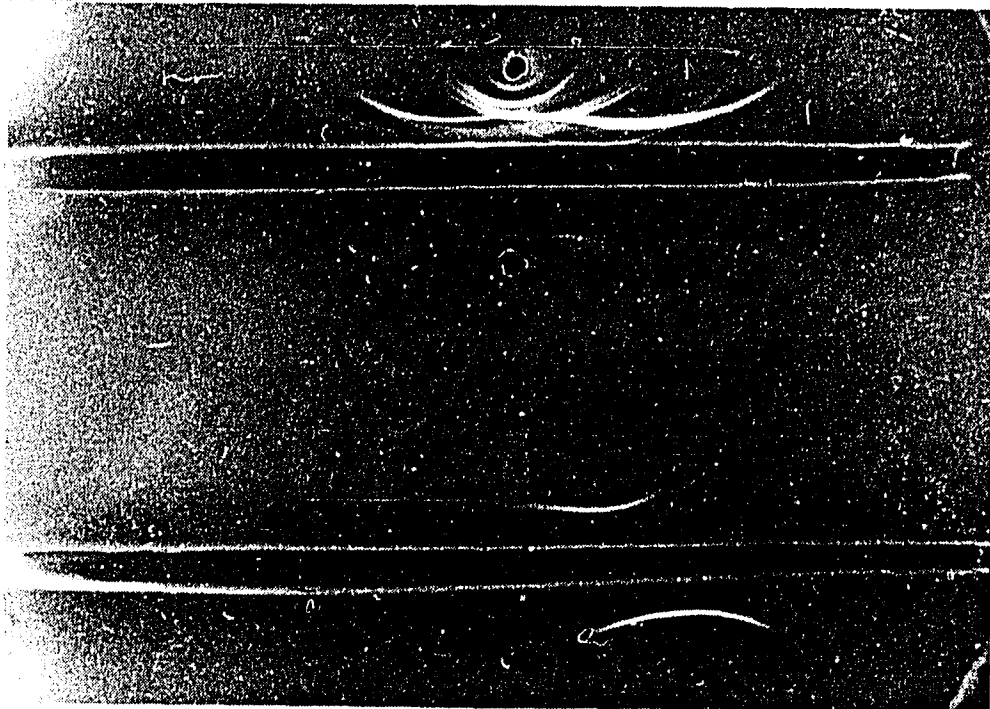


Figure 11. Immunelectrophoretic precipitin pattern of normal porcine serum (NPS); and of IgM, IgA, and IgG. Rabbit anticolostrum serum was placed in the troughs. The anode is at the right.



NPS

IgM

+

IgA

IgG

mobility. Using a batch procedure, proteins were eluted from DEAE Sephadex A50 with buffers of increasing ionic strength. Each eluate exhibited discrete electrophoretic mobilities when tested by cellulose acetate electrophoresis as shown in Figure 12. IgG₂ was eluted with 0.01 M Tris-HCl 0.05 M NaCl buffer pH 8.3 and IgG₁ was eluted with 0.01 M Tris-HCl 0.12 M NaCl buffer pH 8.3. When these fractions were placed in adjacent wells in an agar gel double diffusion and diffused against rabbit-anti porcine IgG, a precipitin spur was observed indicating partial identity.

Column anion exchange chromatography was also performed as a means of obtaining a more purified IgG₂ preparation. The leading edge of a 0.01 M Tris-HCl 0.05 M NaCl buffer pH 8.3 contained pure IgG₂ as shown in Figure 13. Rabbit anti-porcine IgG₂ formed a line of identity with rabbit anti-porcine IgG Fab' 2 and porcine IgG₂, but showed little, if any, precipitin activity with IgG₁ (0.01 M Tris-HCl 0.12 M NaCl buffer pH 8.3 elution).

Estimation of a point of equivalence was performed using agar gel double diffusion (procedure II) as shown in Figure 14.

Sow Passive Immunity Study

A few days after inoculation with T. hyodysenteriae B204 sows 1-6 developed clinical signs of swine dysentery and shed the organism. The data regarding protection are presented in Table 3. Sows 2 and 3 provided solid lactogenic protection to nursing piglets and the pathogen was not shed. Sow 5 provided some protection to her piglets but 27 isolations of T. hyodysenteriae were obtained from 64 swabs. Sows 1 and

Figure 12. IgG-rich anion exchange elution fractions, resolved by cellulose acetate electrophoresis. Samples from left to right are normal porcine serum (NPS), Sepharose 6BCL gel filtration IgG peak of 50% SAS cut of serum (IgG), 0.05 M NaCl (IgG₂), 0.10 M NaCl first wash (1), 0.10 M NaCl second wash (2), 0.12 M NaCl (3), 0.16 M NaCl (4), and 1M NaCl GFB (5). The anode is at the bottom.

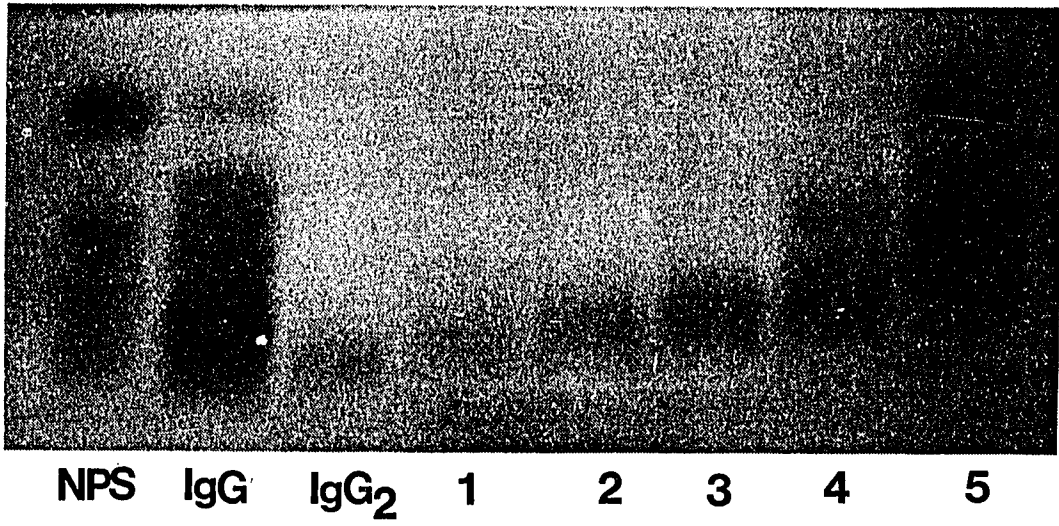


Figure 13. Agar gel double diffusion precipitin patterns formed upon diffusion of porcine IgG₂ (A) and IgG₁-rich fraction (B) against rabbit anti-porcine Fab'2 (C) and rabbit anti-porcine IgG₂ affinity absorbed over IgG₁ (D).

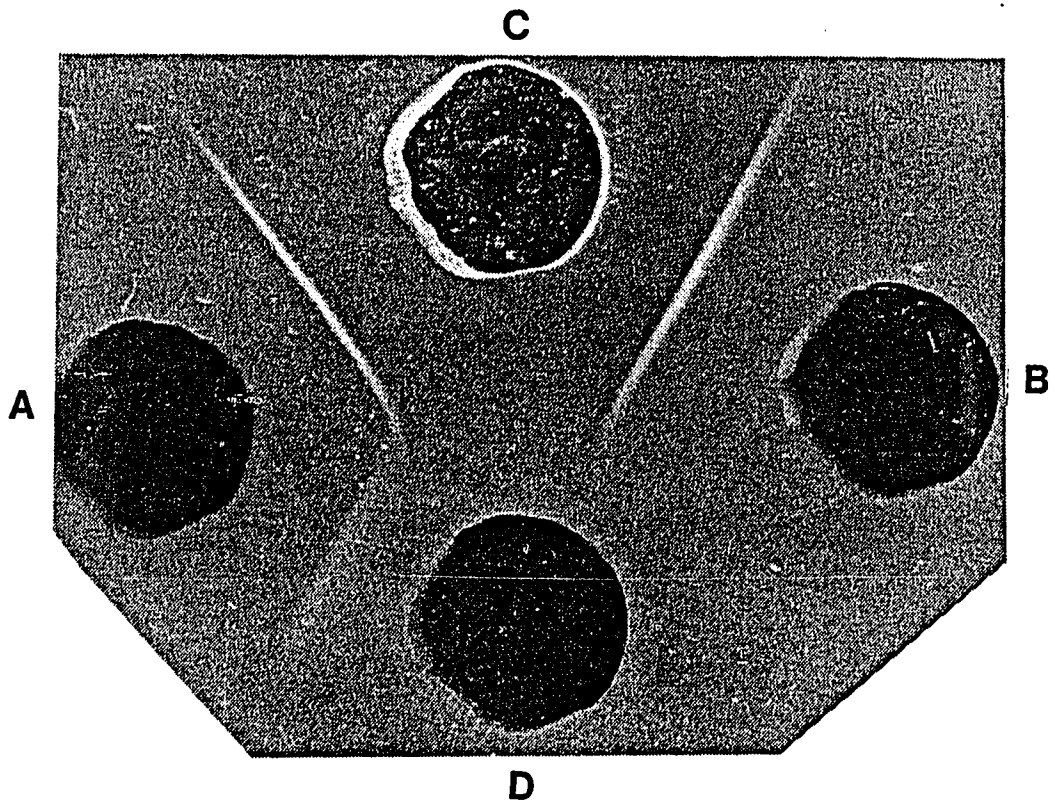


Figure 14. Estimation of point of equivalence using agar gel double diffusion procedure II. The top wells each contain 10 μ l of rabbit anti-alkaline phosphatase. The bottom wells contain 10 μ l of a 1:2 serial dilution of alkaline phosphatase. A sharp precipitin band, zone of equivalence, appears between the six wells from the left.



Table 3. Sow passive immunity to I. hyodysenteriae B204 study. Pluses and minuses indicate time of exposure to I. hyodysenteriae B204, before or after farrowing. The number of isolations of I. hyodysenteriae B204 is presented over the number of swabs taken.

	Exposure		Isolations	
	Sow	Piglets	Before Weaning	After Weaning
Litter No.				
1	+	+	69/72	47/61
2	+	+	0/96	54/88
3	+	+	0/56	27/56
4	+	+	71/80	64/80
5	+	+	27/64	53/62
7	-	+	25/48	21/24
8	-	+	64/64	61/64
9	-	-	0/64	N. D. ^a
10	-	-	0/48	N. D.

^aNot determined.

4 provided negligible protection to their piglets in that 69 isolations were obtained from 71 swabs and 71 isolations from 80 swabs, respectively. Sow 6 had incomplete mammary development and inverted nipples and was removed from the study. Sows 7 and 8 served as controls for pathogenicity of the T. hyodysenteriae and were not exposed to T. hyodysenteriae prior to farrowing; upon postnatal challenge with the pathogen they did not protect their piglets against infection. There were 25 isolations from 48 swabs from piglets from sow 7 and 54 isolations from 64 swabs from piglets from sow 8. Sows 9 and 10 were strict controls and as such these sows and piglets were not exposed to T. hyodysenteriae at any time and T. hyodysenteriae was not isolated from them throughout the study.

Levels of T. hyodysenteriae specific IgA antibodies in the milk from the sows were measured by an amplified-ELISA procedure shown in Figure 15. For this purpose, milk samples were collected from sows 1, 2, 3, and 9 approximately two weeks after farrowing. Antigens reactive with these IgA antibodies were obtained from select isopycnic centrifugation fractions and anion exchange fractions of T. hyodysenteriae. Pooled antigen anion exchange fractions A-1 and A-3 eluted at conductivities of 3.25×10^4 mohs and 5.6×10^4 mohs, respectively. The results of the ELISA test using 10 antigen fractions are presented in Table 4a. Data are presented as the mean O. D. 405 nm response minus the mean O. D. 405 nm response for the control tubes. The mean response for each sow to these 10 antigens are presented in Table 4b. Also, the mean response for each antigen is shown in Table 4c. There is a significant difference ($P < 0.0001$) in level of IgA response to these antigens among the individual sows tested.

Figure 15. Schematic of IgA amplified ELISA procedure.
Antigen is represented as Ag and alkaline
phosphatase as Alk P.

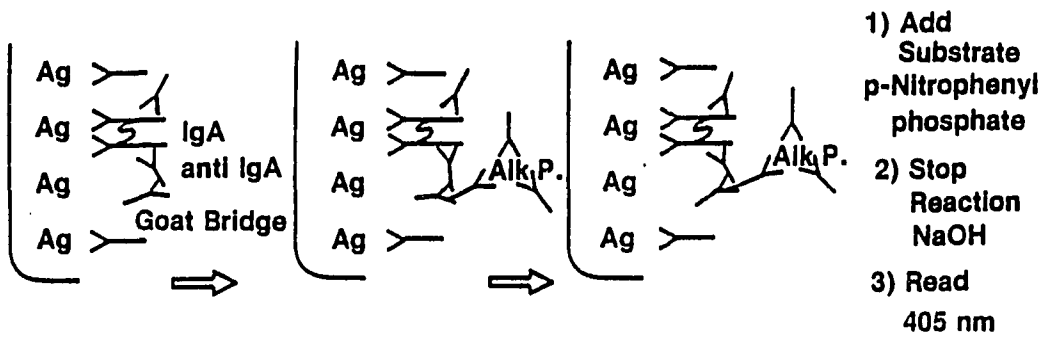
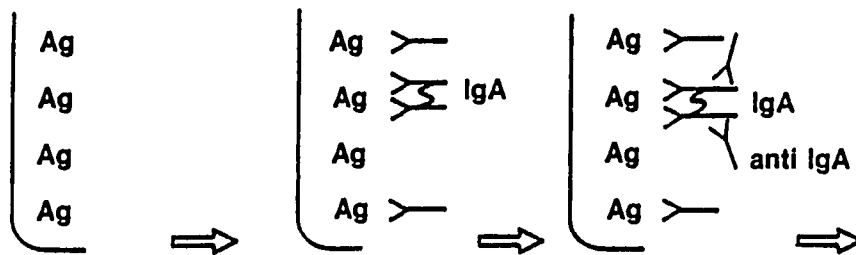


Table 4a. Sow milk IgA ELISA activity (O. D. 405 nm units) to 10 antigen fractions of T. hyodysenteriae B204.

Isopycnic fractions 1-24 and DEAE Sephadex fractions A-1 and A-3									
	1	3	7	10	13	19	24	30	A-1 A-3
Sow									
1	0.202	0.240	0.158	0.335	0.136	0.126	0.170	0.177	0.167 0.260
2	0.256	0.320	0.228	0.315	0.208	0.164	0.170	0.226	0.158 0.318
3	0.908	0.856	0.832	1.206	0.575	0.461	0.537	0.437	0.576 0.582
9	0.312	0.319	0.336	0.515	0.110	0.169	0.930	0.275	0.073 0.192

Table 4b. Mean milk IgA ELISA activity (O. D. 405 nm units) of 10 antigen fractions of T. hyodysenteriae B204 by sows 1-9.

Sow	Antigen	O. D. 405
1	10	0.197
2	10	0.234
3	10	0.697
9	10	0.330

Table 4c. Mean milk IgA ELISA activity (O. D. 405 nm units) of sows 1-9 to 10 antigen fractions of T. hyodysenteriae B204.

Antigen	Sows	O. D. 405
1	4	0.420
2	4	0.447
3	4	0.388
4	4	0.593
5	4	0.257
6	4	0.230
7	4	0.451
8	4	0.279
9	4	0.243
10	4	0.338

Although the response by the sows to the different antigens was not very significant ($P < 0.0593$) when individual antigens were compared, significant differences were observed. The response of sows 2 and 3 which provided protection to their piglets verses the response of sows 1 and 9 which did not provide protection to their piglets was found to be significantly different ($P < 0.005$).

Vaccination Study

In this study, the antigens from a CHAPS extract of *T. hyodysenteriae* B204 were used to vaccinate pigs to determine their role in inducing protection against subsequent oral challenge with live organisms. Fractions that contained *T. hyodysenteriae* specific proteins were chosen as test immunogens. Pigs from all five groups contracted swine dysentery as determined by isolation of *T. hyodysenteriae* from rectal swabs (Table 5). This was supported by observations of clinical signs typical of the disease. Isolation of *T. hyodysenteriae* and the onset of clinical signs in groups 2, 4, and 5 were delayed.

Shown in Table 6a are the group means of weight measurements taken at 4 time intervals during the experiment. Group mean weights were averaged across time and no significant differences were observed in the groups (Table 6b). Although the mean difference in weight between groups 1 and 5 at time 6-24 was 15.75 pounds, the t-test statistic shown in Table 6c does not achieve significance at the 0.05α -level.

Table 5. Isolation of *T. hyodysenteriae* B204 from pigs in vaccination study. Pluses indicate isolation of the organism. Minuses indicate the organism was not isolated.

Group	Pig	4-8	4-14	5-13 ^a	5-17	5-19	5-21	5-24	5-26	5-28	5-31	6-3	6-11	6-24
1	201	-	-	-	-	+	-	-	-	+	+	+	-	-
	202	-	-	-	-	-	+	+	+	+	-	-	-	-
	203	-	-	-	-	-	+	-	-	-	-	-	-	-
	204	-	-	-	-	-	-	-	-	-	-	-	-	-
2	205	-	-	-	-	-	+	-	+	+	-	-	-	-
	206	-	-	-	-	-	-	-	+	+	+	+	-	-
	207	-	-	-	-	-	-	+	-	-	-	-	-	-
	208	-	-	-	-	-	-	+	+	+	-	-	-	-
3	209	-	-	-	+	+	+	+	+	-	-	-	-	-
	210	-	-	-	-	-	-	-	-	-	-	+	-	-
	211	-	-	-	-	-	-	-	+	+	-	-	-	-
	212	-	-	-	-	+	+	-	-	-	-	-	-	-
4	213	-	-	-	-	-	-	+	+	+	-	-	-	-
	214	-	-	-	-	-	-	-	-	+	-	-	-	-
	215	-	-	-	-	-	-	-	+	+	-	-	-	-
	216	-	-	-	-	-	-	-	-	-	-	-	-	-
5	217	-	-	-	-	-	-	-	-	+	+	-	-	-
	218	-	-	-	-	-	-	-	+	+	+	-	+	-
	219	-	-	-	-	-	-	+	+	+	+	-	-	-
	220	-	-	-	-	-	-	-	+	+	+	-	-	-

^aDate of initial exposure

Table 6a. Pig group mean weight measurements (pounds) of vaccination study by date.

	4-7	5-13 ^a	5-28	6-24
Group				
1	37.75	79.50	113.50	150.75
2	37.75	69.00	91.50	159.50
3	38.00	81.25	104.00	150.00
4	38.00	76.25	100.50	149.50
5	36.50	72.25	97.50	166.50

^aDate of initial exposure to I. hyodysenteriae.

Table 6b. Test of group differences.

	df	mean square	F(4,15)
Between groups	4	83.36	1 N. S. ^a
Within groups	15	216.6	

^aNot significant.

Table 6c. t-test statistic for the mean difference between groups 1 and 5.

$$t_T = 15.75 / \sqrt{2(216.6)/4} = 1.5$$

DISCUSSION

The goal of this study was to isolate T. hyodysenteriae specific proteins that could be used as immunogens or as diagnostic antigens. An extraction procedure that would gently remove surface antigens of T. hyodysenteriae yet leave the organism's protoplasmic cylinder containing complex metabolic enzymes intact was desired.

CHAPS, a zwitterionic derivative of cholic acid recently developed by Hjelmeland (1980), was chosen. This detergent has been shown to be very effective in solubilizing membrane bound proteins without denaturation (Simonds et al., 1980). CHAPS has an extremely small micelle molecular weight and a high critical micelle concentration (Hjelmeland and Chramback, 1981). It has several advantageous characteristics in that it can be easily removed from gels and it does not interfere with isoelectric focusing, ion exchange chromatography or U. V. absorption.

CHAPS effectively removed the outer envelope of the organism, which contained a complex mixture of proteins that included at least two which were T. hyodysenteriae specific. These specific proteins were initially detected by agar gel double diffusion with affinity absorbed rabbit anti-T. hyodysenteriae serum.

The T. innocens activity absorbed from rabbit anti-T. hyodysenteriae serum depended on the type of affinity column used. Two different coupling procedures and support media were used to covalently attach an extract of T. innocens for affinity chromatography. Each column appeared to be more effective in removing particular shared, but different, treponemal activity. The covalent linkage procedure in each coupling

reaction may have deactivated or blocked certain antigenic sites inhibiting absorption.

Once these T. hyodysenteriae specific proteins were identified, the next objective was to isolate them. Centrifugation of this complex mixture through a linear sucrose gradient to equilibrium separated the proteins into smaller groups of defined buoyant density. Isopycnic centrifugation in a vertical tube rotor was found to be a convenient preparative method of dividing this mixture into less complex workable units.

SDS PAGE was an effective tool for resolving T. hyodysenteriae specific proteins present in fractions of the isopycnic centrifugation and immune precipitates of pathogenic T. hyodysenteriae B204. These specific proteins were not seen in immune precipitates of nonpathogenic T. innocens B256.

The molecular weights of specific T. hyodysenteriae proteins were determined on the assumption that every protein binds 1.2 g SDS per g protein (Reynolds and Tanford, 1970). However, with glycoproteins, lipoproteins, and membrane proteins this proportional binding premise may not always be accurate (Wyckoff et al., 1977). Also, sample treatment with 2-mercaptoethanol and SDS prior to gel electrophoresis presumably reduced all intra and inter disulfide bonds. Potential peptide associations were not determined.

Two dimensional gel electrophoresis was conducted on a CHAPS extract of T. hyodysenteriae. Clearly, this procedure can be used for characterization of treponemal antigens. A more sensitive method of detection such

as a silver stain or an isotopic probe together with computer enhanced resolution would be helpful if this technique is repeated. Molecular weight and isoelectric point determination of these proteins would aid in their purification.

At the time this study was initiated, porcine immunoglobulin reagents of satisfactory specificity were not commercially available. Early work was directed toward purification of classes and subclasses of porcine immunoglobulins and production of heavy chain specific antisera.

Pentameric IgM was isolated from an euglobulin precipitate of porcine serum by gel filtration. Alpha-2 macroglobulin is a common contaminant of IgM preparations. To prevent this, only the leading edge of the IgM gel filtration peak was pooled. Isoelectric point precipitation removed most, but not all, of the alpha-2 macroglobulin.

Dimeric IgA was collected by gel filtration of an SAS precipitation of milk. This fraction also contained alpha-2 macroglobulin, the bulk of which was removed by isoelectric point precipitation. Admittedly, this preparation did contain traces of IgG. Its removal was not necessary in order to produce specific rabbit anti-IgA. This antiserum did not react with IgG or IgM by agar gel double diffusion.

A myeloma has not been described in the porcine species, thus immunoglobulin subclass characterization has not progressed as rapidly as in the mouse and human. Production of porcine heavy chain specific IgG₂ has not been previously reported. In this study, porcine IgG₂ specific antiserum was produced. Although this antiserum was not used to monitor porcine IgG₂ specific I. hyodysenteriae activity, it may have

useful diagnostic application in studying immunologic responses through the course of infections.

Fundamental information on the mechanism of resistance to *T. hyodysenteriae* was desired. It was not known whether or not humoral or cell mediated immunity or a combination of both is the primary means of resistance to swine dysentery. In many other enteric infections, including TGE (Bohl et al., 1972) and colibacillosis (Porter and Chidlow, 1979), IgA has been shown to play a crucial role in neonatal defense. Also, it has been observed that nursing piglets tend not to show clinical signs of swine dysentery until the onset of weaning (Harris and Glock, 1981).

There is a definite link between intestinal immune response and IgA antibody response by the mammary gland. In mice, it has been shown that intestinal mucosal lymphocytes will migrate and lodge in mammary associated lymphoid tissue (Roux et al., 1977). It is believed that a similar lymphocyte migration occurs in swine (Porter and Chidlow, 1979).

A study was designed to determine if dysentery recovered sows passively protect their piglets. Results indicated that in at least two litters, milk alone provided complete protection against oral challenge with the organism until weaning. Sow milk, in addition to being a rich source of IgA, does not have the inherent collection and storage problems associated with intestinal secretions.

An in vitro assay, the amplified ELISA, was designed to detect *T. hyodysenteriae* specific IgA antibody in milk. Levels of milk IgA activity to two antigen fractions, isopycnic centrifugation fraction 13

buoyant density 1.130 g/ml and DEAE A50 fraction A-3, gave a higher response to sows that provided protection to their piglets than to sows that did not provide protection to their piglets.

The serum agglutinating antibodies may not be indicative of protection. The piglets in this previous sow study which had high serum agglutinating responses were not protected from oral challenge with the organism (Dr. D. L. Harris, Dept. Vet. Micro., Iowa State Univ., Ames, IA., personal comm., 1981). Apparently, colostrum-derived serum antibodies were ineffective in inhibiting colonization of T. hyodysenteriae in the nursing piglet. Levels of IgA show a higher correlation with protection. Although attachment of T. hyodysenteriae to the intestinal epithelium has not been conclusively shown, IgA may function by blocking attachment and/or preventing colonization.

Since it was known that there was an association between IgA activity and protection against swine dysentery, T. hyodysenteriae specific proteins were selected as potential immunogens in a vaccine study. An intraperitoneal route of immunization was used in an attempt to induce a protective IgA response. This route of initial immunization has been shown to stimulate an IgA response in pigs (Bennell and Husband, 1981b). The immune complex group weight gain was significantly greater than the control group. It is possible that these pigs were better able to fight off the disease because of the presence of secretory IgA T. hyodysenteriae specific antibody.

SUMMARY

The initial objective of this study was to isolate T. hyodysenteriae specific proteins that may be involved in inducing immune protection. It was thought T. hyodysenteriae specific proteins may serve as useful diagnostic antigens as well as potential immunogens.

A study was conducted to determine whether or not swine dysentery-recovered sows would provide protection to their piglets, via milk, from oral challenge with live T. hyodysenteriae organisms. Therefore, sows were infected with T. hyodysenteriae during the later stages of gestation to optimize antibody production by the mammary gland.

It appears that piglets acquire passive protection against swine dysentery from milk. Clinical signs of swine dysentery and isolation of T. hyodysenteriae usually occurred after weaning. Thus, immunoglobulins present in the milk may provide sole protection to swine dysentery by inhibition of T. hyodysenteriae colonization in the large intestine.

Although much has been published about the classes of porcine immunoglobulins, little is known about the subclasses. In this study, a method of purification of IgG₂ and production of heavy chain specific antiserum was described.

CHAPS detergent, shown to be effective in solubilizing membrane bound proteins without denaturation, removed only the outer envelope of the organism as seen in the electron micrograph. This extraction removed a complex mixture which appeared to be proteineous in nature and could be easily precipitated with increasing concentration of ammonium sulfate.

Isopycnic centrifugation separated this extract into fractions of less complexity.

T. hyodysenteriae specific proteins were initially identified by agar gel double diffusion with antiserum produced in rabbits against an NP40 extract of T. hyodysenteriae. Absorption of this antiserum over an affinity column prepared by covalently attaching T. innocens to a cross linked agarose column removed much of its shared activity with T. hyodysenteriae and T. innocens. A second T. innocens affinity column prepared using a different support bed and covalent linking procedure appeared effective at removing shared treponemal activity not removed by the agarose column.

Cross immunoelectrophoresis resolved precipitin bands not seen by agar gel diffusion. This procedure, although tedious, may prove to be a useful tool in describing antigens of T. hyodysenteriae.

Two dimensional gel electrophoresis was conducted on a CHAPS extract of T. hyodysenteriae. With a more sensitive detection system, this procedure has tremendous potential for resolving treponemal antigens.

An enzyme-linked immune assay (amplified ELISA) was developed to determine if IgA was involved in resistance to swine dysentery. Various components of T. hyodysenteriae were assayed for IgA activity in the milk. Fraction 13 (buoyant density 1.130 mg/g) and a basic protein fraction from DEAE Sephadex A50 appeared to give a higher response to sows that provided protection to their piglets than to sows that did not provide protection to their piglets.

Specific proteins of T. hyodysenteriae initially identified by agar

gel double diffusion in fractions 3 and 13 (buoyant density 1.165 g/ml and 1.130 g/ml, respectively) were also resolved by SDS PAGE of immunoprecipitates of T. hyodysenteriae.

A vaccine study was attempted to find a method of stimulating a protective IgA response using these fractions containing T. hyodysenteriae specific proteins as immunogens. An intraperitoneal route of immunization was used. Although every group shed the organism, the group that received an immunoprecipitate form with affinity absorbed rabbit anti-T. hyodysenteriae B204 and a CHAPS extract of T. hyodysenteriae B204 had 15.75 pounds greater weight gain than the control group. This weight gain, however, was found not to be significant.

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